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| <p>(54) Title: ANGIOGENIC MODULATION BY NOTCH SIGNAL TRANSDUCTION</p> <p>(57) Abstract</p> <p>This invention provides methods of modulating angiogenesis, including promoting or inhibiting angiogenesis, e.g., in connection with treating abnormalities including hemangiomas, hemangiosarcomas, Kaposi's Sarcoma, ischemic disorders, and wounds. These methods involve administration of compounds that are selective agonists or antagonists of Notch4 protein. In addition, this invention provides an isolated nucleic acid molecule encoding a Notch4, an isolated Notch4 protein, vectors comprising an isolated nucleic acid molecule encoding a Notch4 protein, cells comprising such vectors, antibodies directed to Notch4 protein, nucleic acid probes useful for detecting nucleic acids encoding Notch4 protein, and antisense oligonucleotides complementary to any unique sequences of a nucleic acid molecule which encodes Notch4 protein.</p> | | |

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ANGIOGENIC MODULATION BY NOTCH SIGNAL TRANSDUCTION

5 The invention disclosed herein was made with Government support under Grant Nos. DAMD17-94-J-4410, DAMD17-94-J-4153 and DAMD17-97-1-7291 from the Department of Defense. Accordingly, the U.S. Government has certain rights in this invention.

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Background of the Invention

Throughout this application, various publications are referenced by author and date. Full citations for these publications may be found listed alphabetically at the end of the specification immediately preceding the claims. The disclosures of these publications in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art as known to those skilled therein as of the date of the invention described and claimed herein.

The int-3 gene was originally identified based on its oncogenic effects in the mouse mammary gland. int-3 is a frequent target for insertional activation by MMTV proviral DNA in MMTV-induced mammary gland tumors (Gallahan and Callahan, 1987; Robbins et al., 1992; Sarkar et al., 1994). Tumor specific transcripts derived from the int-3 gene encode a protein homologous to the intracellular part of the Notch family of cell surface receptors. Exogenous expression of the int-3 oncoprotein has been shown to affect the growth and development of mammary epithelial cells. Over expression of the int-3 oncoprotein in a mouse mammary epithelial cells (HC11) promotes anchorage independent growth (Robbins et al., 1992). Expression of int-3 as an MMTV-LTR driven transgene in the mouse mammary gland results in abnormal development of the mammary gland and rapid development of undifferentiated mammary carcinomas (Jhappan et al., 1992). In the normal mouse mammary gland, endogenous

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int-3 protein has been detected in mammary stroma and epithelium (Smith et al., 1995).

Members of the Notch/lin-12 gene family were first
5 identified in Drosophila and Caenorhabditis elegans
through genetic analysis of mutations that alter cell
fate decisions (for review see Artavanis-Tsakonas et al.,
1995; Artavanis-Tsakonas and Simpson, 1991; Greenwald and
Rubin, 1992). Drosophila Notch regulates multiple cell
10 fate decisions that involve cell-cell interactions during
fly development, for instance, control of cell fate
decisions involving neural/epidermal specification in
proneural clusters (Artavanis-Tsakonas and Simpson,
1991). The C. elegans lin-12 and glp-1 proteins are
15 structurally related to Notch, and are also involved in
cell fate specifications during development in the
nematode (Greenwald, 1985; Yochem and Greenwald, 1989).
Genetic analysis of Notch/lin-12 genes suggest that this
family of genes controls binary cell fate decisions and
20 inductive signaling that depend on cell-cell interactions
(reviewed in Artavanis-Tsakonas et al., 1995; Greenwald,
1994; Greenwald and Rubin, 1992). Alternatively,
Notch/lin-12 genes have been proposed to block cell
differentiation, thus maintaining the competence of cells
25 for subsequent cell-fate determination (Coffman et al.,
1993; Fortini et al., 1993).

Notch/lin-12 genes encode transmembrane receptor proteins
characterized by highly repeated, conserved domains. The
30 amino terminus of Notch proteins encodes the
extracellular domain and contains as many as 36 repeats
of an EGF-like motif involved in ligand binding (Rebay et
al., 1993), and three tandem copies of a Notch/lin-12
sequence motif of unknown function. The intracellular
35 portion of Notch proteins is characterized by six tandem
copies of a cdc10/ankyrin motif, thought to be a protein-
protein interaction domain (Michaely and Bennett, 1992),

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and a PEST sequence motif which may represent a protein degradation signal (Rogers et al., 1986). In several systems, truncated forms of Notch/lin-12 proteins that contain an intact intracellular domain without most of the extracellular domain behave as constitutively activated receptors (reviewed in Artavanis-Tsakonas et al., 1995; Greenwald, 1994). The human Notch 1 orthologue, TAN-1, was first identified in independently isolated translocation breakpoints in acute T lymphoblastic leukemia and is predicted to encode a truncated product that has an intact intracellular domain but lacks most of the extracellular domain (Ellisen et al., 1991). Similarly, the int-3 oncoprotein encodes the intracellular domain of a Notch-like protein and thus has been proposed to act as an activated Notch receptor (Robbins et al., 1992).

Based on sequence similarity to Drosophila Notch, additional Notch-related genes have been isolated from mammals; including mouse (Franco Del Amo et al., 1993; Lardelli et al., 1994; Lardelli and Lendahl, 1993; Reaume et al., 1992), rat (Weinmaster et al., 1992; Weinmaster et al., 1991), and human (Ellisen et al., 1991; Stifani et al., 1992; Sugaya et al., 1994). To date, three Notch homologues, Notch1, Notch2, and Notch3, have been identified in the mouse and their embryonic expression patterns display partially overlapping but distinct patterns of expression that are consistent with a potential role in the formation of the mesoderm, somites, and nervous system (Williams et al., 1995). Abundant expression of Notch1, Notch2, and Notch3 is found in proliferating neuroepithelium during central nervous system development. Targeted disruption of the Notch1 gene in mice results in embryonic death during the second half of gestation (Conlon et al., 1995; Swiatek et al., 1994) and homozygous mutant embryos display delayed somitogenesis as well as widespread cell death,

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preferentially in neuroepithelium and neurogenic neural crest (Conlon et al., 1995; Swiatek et al., 1994).

5 The gene products of Drosophila Delta (Vassin et al., 1987) and Serrate (Fleming et al., 1990), and C. elegans
Lag-2 (Henderson et al., 1994; Tax et al., 1994) and Apx-1 (Mello et al., 1994) are thought to act as ligands for
Notch proteins. In the mouse, the orthologue of Delta,
10 referred to a Dll1 (Delta-like gene 1), is expressed
during embryonic development in the paraxial mesoderm and
nervous system in a pattern similar to that of mouse
Notch1 (Bettenhausen et al., 1995). A murine Serrate-
related gene named Jagged has been identified and is
partially co-expressed with murine Notch genes in the
15 developing spinal cord (Lindsell et al., 1995).

The identification and expression analysis of a fourth
murine Notch homologue is reported here. The fourth
murine Notch homologue has been named Notch4 and the int-
20 3 nomenclature has been reserved for the truncated
oncogene. Although the intracellular domain of the int-3
oncoprotein shares homology with the Notch/Lin-12 protein
family, a comparison of the full length Notch4 protein to
that of the int-3 oncoprotein is now provided. The
25 activated int-3 protein encodes only the transmembrane
and intracellular domain of the Notch4 protein. The
predicted amino acid sequence of Notch4 contains the
conserved features of all Notch proteins, however Notch4
has 7 fewer EGF-like repeats compared to Notch1 and
30 Notch2 and contains a significantly shorter intracellular
domain. Notch4 is expressed primarily in embryonic
endothelium and in adult endothelium and male germ cells.

This invention uses an established cell line, Rat Brain
35 Microvessel Endothelial cells (RBE4 cells), to test
Notch4 activity and to demonstrate that activated Notch4
and Notch4 ligand stimulate angiogenesis in these cells.

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RBE4 cells grown on collagen coated plates in the absence of bFGF or with low concentrations of bFGF (1ng/ml), display a cobblestone morphology. In the presence of high concentration of bFGF (5ng/ml), RBE4 cells exhibit a spindle shape morphology. When RBE4 cells reach confluency, they growth arrest and cells retain their cobblestone morphology. Post-confluent RBE4 cell cultures grown in the presence of 5ng/ml bFGF, develop multicellular aggregates from the cobblestone monolayer. These three-dimensional structures are sprouts that extend above the monolayer and some of these sprouts will organize into curvilinear and bifurcating structures that float in the cell culture medium. The RBE4 cell sprouts and three-dimensional structures contain high activity of several enzymatic markers that are specific for differentiated microvessels. Thus, bFGF, a known angiogenic agent, induces angiogenesis of RBE4 cells which upon treatment with high concentrations of bFGF develop structures resembling capillaries (based on their morphological appearance and based on their expression of differentiated endothelial cell markers).

The Notch1 and Jagged genes have been previously described to be expressed in mouse endothelial cells.

Contrary to previous studies suggesting that Jagged and Notch inhibit angiogenesis (Zimrin et al. J Biol Chem. 1996), this invention demonstrates that activated Notch4 protein and Jagged protein contain a biological activity on RBE4 cells that is similar to the known angiogenic agent basic fibroblast growth factor (bFGF), suggesting that the above-described biological activity is an angiogenic activity.

This invention demonstrates that RBE4 cells express an endogenous Notch, since RBE4 cells that are programmed to express Jagged (a mammalian Notch ligand) have a similar

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phenotype when compared to RBE4 cells that express a constitutive activated Notch (int-3). This is consistent with previously published data showing that Notch4 is expressed in endothelial cells in vivo.

5

This invention demonstrates that RBE4 cells do not express sufficient levels of Notch4 ligand to activate the Notch4 receptor, since RBE4 cells programmed to express Notch4 do not exhibit a spindle form morphology when grown in the absence of bFGF.

10

This invention also demonstrates that over expression of Notch4 protein in RBE4 cells does not result in activation of the Notch4 receptor, because otherwise one would expect that these cells would have a similar phenotype to those cells that express a constitutive activated form of the Notch4 receptor.

15

This invention also demonstrates that bFGF mediated angiogenesis is unrelated to Notch4 mediated angiogenesis. This invention further suggests that Jagged or activated Notch4 induced activity can synergize or cooperate with bFGF.

20

Notch signaling is typically associated with cell fate decision. The finding that Notch activation stimulates capillary outgrowth suggests that Notch receptors must be activated to allow this process to occur. Therefore, Notch modulation provides a novel paradigm for regulating angiogenesis. Specifically, modulation of Notch signaling can be used to modulate angiogenesis either positively, by activating Notch signaling to stimulate angiogenesis or negatively, by blocking Notch signaling to block angiogenesis. This modulation would be distinct from previous proteins known to modulate angiogenesis. The induction or inhibition of angiogenesis in vivo can be used as a therapeutic means to treat a variety of

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diseases, including but not limited to cancer, diabetes, wound repair and arteriosclerosis.

5 This invention provides three major advantages over previously published paradigms. First, because Notch signaling is distinct from FGF signaling, this offers a different strategy to affect angiogenesis that may or may not be more effective than FGFs. Second, because Notch signaling establishes cell fate decision, by blocking
10 Notch, angiogenesis may be blocked regardless of what other angiogenic factors are present. Third, Notch and FGF may be used synergistically to modulate angiogenesis.

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Summary of the Invention

This invention provides an isolated nucleic acid molecule,
encoding a Notch4 protein. This invention provides a
5 vector comprising the above-described nucleic acid. This
invention also provides an isolated Notch4 protein.

This invention also provides a plasmid which comprises the
regulatory elements necessary for expression of DNA in a
10 mammalian cell operatively linked to the DNA encoding
Notch4 protein so as to permit expression thereof.

This invention also provides a mammalian cell comprising
the above-described plasmid or vector.

15 This invention also provides a nucleic acid probe
comprising a nucleic acid of at least 12 nucleotides
capable of specifically hybridizing with a unique sequence
included within the sequence of a nucleic acid encoding
20 Notch4 protein.

This invention also provides an antisense oligonucleotide
having a sequence capable of specifically hybridizing to
mRNA encoding Notch4 protein so as to prevent translation
25 of the mRNA.

This invention also provides an antisense oligonucleotide
having a sequence capable of specifically hybridizing to
mRNA encoding activated Notch4 protein so as to prevent
30 translation of the mRNA.

This invention provides an antisense oligonucleotide having
a sequence capable of specifically hybridizing to the
genomic DNA encoding a Notch4 protein or an activated
35 Notch4 protein.

This invention provides an antibody directed to a Notch4

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protein. This invention provides a pharmaceutical composition comprising an amount of the oligonucleotide effective to reduce activity of Notch4 protein by passing through a cell membrane and binding specifically with mRNA
5 encoding Notch4 protein in the cell so as to prevent its translation and a pharmaceutically acceptable carrier capable of passing through a cell membrane.

This invention provides a pharmaceutical composition
10 comprising an amount of an antagonist effective to reduce the activity of Notch4 protein and a pharmaceutically acceptable carrier.

This invention provides a pharmaceutical composition
15 comprising an amount of an agonist effective to increase activity of Notch4 protein and a pharmaceutically acceptable carrier.

This invention provides the above-described pharmaceutical
20 composition which comprises an amount of the antibody effective to block binding of a ligand to the Notch4 protein and a pharmaceutically acceptable carrier.

This invention also provides a method for determining
25 whether a ligand can specifically bind to Notch4 protein comprising the steps of: a) contacting Notch4 protein with the ligand under conditions permitting formation of specific complexes between Notch4 protein and known Notch4 protein-binding ligands; b) determining whether complexes
30 result from step (a), the presence of such complexes indicating that the ligand specifically binds to Notch4 protein.

This invention provides a method for determining whether a
35 ligand can specifically bind to a Notch4 protein which comprises preparing a cell extract from cells transfected with and expressing DNA encoding the Notch4 protein,

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isolating a membrane fraction from the cell extract,
contacting the membrane fraction with the ligand under
conditions permitting binding of ligands to such Notch4
protein, detecting the presence of the ligand specifically
5 bound to Notch4 protein, and thereby determining whether
the ligand specifically binds to Notch4 protein.

This invention provides a method for determining whether a
ligand is a Notch4 protein agonist which comprises
10 contacting a cell transfected with and expressing nucleic
acid encoding Notch4 protein with the ligand under
conditions permitting activation of a functional Notch4
protein response by ligands known to be agonists of Notch4
protein, and detecting whether a functional increase in
15 Notch4 protein activity occurs so as to determine whether
the ligand is a Notch4 agonist.

This invention provides a method for determining whether a
ligand is a Notch4 protein antagonist which comprises
20 contacting a cell transfected with and expressing DNA
encoding Notch4 protein with the ligand under conditions
permitting the activation of a functional Notch4 protein,
and detecting whether a functional decrease in Notch4
activity occurs so as to determine whether the ligand is a
25 Notch4 protein antagonist.

This invention provides a method of treating an abnormality
in a subject, wherein the abnormality is alleviated by the
inhibition of Notch4 protein activity which comprises
30 administering to a subject an amount of Notch4 antagonist
effective to inhibit Notch4 protein activity.

This invention provides a method of treating an abnormality
in a subject wherein the abnormality is alleviated by the
35 activation of Notch4 activity which comprises administering
to a subject an amount of a Notch4 agonist effective to
promote Notch4 protein activity.

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This invention provides a method of preparing isolated Notch4 protein which comprises: a) inserting nucleic acid encoding Notch4 protein in a suitable vector which comprises the regulatory elements necessary for expression
5 of the nucleic acid operatively linked to the nucleic acid encoding Notch4 protein; b) inserting the resulting vector in a suitable host cell so as to obtain a cell which produces Notch4 protein; c) recovering the Notch4 protein produced by the resulting cell; and d) purifying the
10 protein so recovered.

This invention also provides a method of modulating angiogenesis in a subject comprising administering to the subject an effective amount of agonist or antagonist of the
15 Notch4 protein so as to promote or inhibit angiogenesis in the subject.

This invention also provides a method of promoting angiogenesis in a subject comprising administering to the
20 subject an amount of Notch4 protein agonist effective to promote angiogenesis in the subject.

This invention also provides method of inhibiting angiogenesis in a subject comprising administering to the
25 subject an amount of Notch4 protein antagonist effective to inhibit angiogenesis in the subject.

This invention also provides method of promoting angiogenesis comprising transducing selected cells, wherein
30 the cells express activated Notch4 protein in an amount sufficient to promote angiogenesis in the cells.

This invention provides a method of promoting angiogenesis comprising transducing selected cells which express Notch4
35 protein, wherein the cells express a Notch4 ligand in an amount sufficient to promote angiogenesis in the cells.

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This invention provides a method of promoting angiogenesis comprising transducing selected cells, wherein the cells express a Notch4 protein and a Notch4 ligand in an amount sufficient to promote angiogenesis in the cells.

5

This invention provides a method of inhibiting angiogenesis comprising administering to cells expressing Notch4 protein an amount of a specific antibody effective to block binding of a ligand to Notch4 protein.

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This invention provides a method of inhibiting angiogenesis comprising administering to cells expressing Notch4 protein an amount of a fragment of a specific antibody effective to block binding of a ligand to Notch4 protein.

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This invention provides a method of treating an abnormality in a subject wherein the abnormality is alleviated by inhibiting angiogenesis comprising blocking Notch4 protein signaling in the subject.

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This invention also provides a method of treating an abnormality in a subject wherein the abnormality is alleviated by promoting angiogenesis comprising stimulating the Notch4 signaling pathway.

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Brief Description of the Figures

Figure 1 Deduced amino acid sequence of Notch4 (GenBank accession number U43691). The boxed regions indicate the major structural elements of the Notch family of proteins, as follows: 29 epidermal growth factor (EGF)-like repeats; 3 Notch/lin12 repeats; a transmembrane domain; and 6 cdc10/ankyrin repeats. Putative glycosylation sites are underlined. A putative PEST domain is doubly underlined. The two cysteines thought to promote dimerization are marked with asterisks. The initiating methionine of the int-3 oncoprotein is in bold and marked by an arrow.

Figure 2 Schematic structural comparison of the four murine Notch proteins. The EGF-like repeats are numbered according to their position in each different protein. Where equivalent EGF-like repeats can be identified, connecting lines are placed to compare the relationship between these repeats in different Notch proteins (see EGF-repeat equivalents). Notch4 contains seven EGF-like repeats, fewer than Notch1 and Notch2. One of the missing EGF-like repeats (#25) in Notch4 is derived from equivalent repeats #31 and #32 of Notch1/Notch2, creating a novel and hybrid EGF-like repeat. Eight of the EGF-like repeats of Notch4 (#14 to #21) have no identifiable equivalent repeats in Notch1/Notch2. The region of Notch4 from the end of the cdc10/ankyrin repeats to the carboxy terminus is shorter when compared to Notch1, 2 and 3.

Figure 3 Amino acid sequence comparison of EGF-like repeat #11 of mouse Notch1, 2, 3 and 4. Residues conserved between the mouse Notch proteins are shaded and the putative calcium-binding sites are marked with arrows. A region within EGF-like repeat #11 of the Notch proteins containing non-conserved and variable numbers of residues is boxed. The leucine to proline mutation in Xenopus Notch that obliterates binding to Delta is marked with an

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asterisk (*).

Figure 4 Expression analysis of Notch4 in adult mouse tissues. (A) Northern blot using a riboprobe transcribed from the 3' UTR of Notch4 (probe D in Fig. 5). (B) The same blot reprobed with a GAPDH probe. The transcript sizes of 6.7 kb, 1.5 kb and 1.1 kb are indicated and were estimated with reference to 28S and 18S rRNA migration.

Figure 5 Expression analysis of Notch4 testis transcripts. (A) Notch4 testis transcripts are expressed in post-meiotic germ cells. Northern blot analysis from staged and germ cell-deficient testes with probe C and a GAPDH probe. Note that GAPDH transcripts appear as two isoforms in the adult testis. RNA was isolated from testes of day 7 p.n., day 17 p.n., adult, W^y/W and W/+ mice, as indicated. (B) Northern blot analysis of several adult tissues with probe A, derived from the 5' UTR of Notch4 and a GAPDH probe. (C) Schematic representation of truncated Notch4 transcripts as compared to the full-length coding potential. Relative positions of probes used in the northern blot analysis are shown. Conserved elements of Notch Family proteins are indicated. The MMTV integration site reported by Robbins et al. (1992) is indicated by an arrow. Novel 5' sequences of testes cDNAs are indicated.

Figure 6 Notch4 is expressed in embryonic endothelial cells. (A,B) Phase contrast and dark-field photomicrograph of a horizontal section of a 9 d.p.c. embryo hybridized with a cRNA probe corresponding to Notch4. Strong labeling is detectable over the anterior cardinal vein (white/black arrows). Diffuse labeling is also present throughout the developing nervous system and at higher levels over the tip of the neural folds (red arrows). (C-F) Phase and darkfields images of a horizontal section of a 13.5 d.p.c. embryo hybridized for Notch4, showing the venous and arterial system anterior to the lung, including dorsal

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aorta arch, aortic and pulmonary tract. E and F are higher magnifications of the area framed in C. Embryonic vessels are labeled and, as shown in E and F, labeling is restricted to the endothelial cells lining the vessels. Arrows denote the gut, which does not have a detectable signal in the epithelium.

Figure 7 Notch4 is expressed in adult lung endothelial cells. (A, B) Phase contrast and darkfield photomicrographs of an adult mouse lung hybridized with a cRNA probe corresponding to Notch4. Punctate staining is observed over the alveolar walls, which are predominantly composed of capillaries. No labeling is observed over the pseudostratified squamous epithelium (black and white arrows) nor over the smooth muscle cells (red arrows).

Figure 8 RBE4 cell lines grown on collagen coated plates in the absence of bFGF. (A) RBE4/LacZ, low magnification, 1/8 sec exposure; (B) RBE4/LacZ, high magnification, 1/8 sec exposure; (C) RBE4/LacZ, low magnification, 1/4 sec exposure; (D) RBE4/LacZ, high magnification, 1/4 sec exposure; (E) RBE4/Notch4, low magnification, 1/8 sec exposure; (F) RBE4/Notch4, low magnification, 1/4 sec exposure; (G) RBE4/Notch4, high magnification, 1/8 sec exposure; (H) RBE4/Notch4, high magnification, 1/4 sec exposure; (I) RBE4/Jagged, low magnification, 1/8 sec exposure; (J) RBE4/Jagged, low magnification, 1/8 sec exposure; (K) RBE4/Jagged, low magnification, 1/4 sec exposure; (L) RBE4/Jagged, low magnification, 1/4 sec exposure; (M) RBE4/Jagged, high magnification, 1/8 sec exposure; (N) RBE4/Jagged, high magnification, 1/8 sec exposure; (O) RBE4/Jagged, high magnification, 1/4 sec exposure; (P) RBE4/Jagged, high magnification, 1/4 sec exposure; (Q) RBE4/int-3, low magnification, 1/8 sec exposure; (R) RBE4/int-3, low magnification, 1/8 sec exposure; (S) RBE4/int-3, low magnification, 1/4 sec exposure; (T) RBE4/int-3, low magnification, 1/4 sec exposure.

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exposure; (U) RBE4/int-3, high magnification, 1/8 sec exposure; (V) RBE4/int-3, high magnification, 1/8 sec exposure; (W) RBE4/int-3, high magnification, 1/4 sec exposure; (X) RBE4/int-3, low magnification, 1 / 4 s e c exposure.

Figure 9 Nucleic acid sequence of Notch4 (GenBank Accession No. U43691)

Fig 10 Immunohistochemical analysis on adult mouse kidney sections. Endothelial cells within the cortical kidney glomeruli can be detected by using either an anti-PECAM antibody (B) or an antibody was used in panel B, and both panel A and B were processed for horse radish peroxidase detection which results in a brown staining. Pre-immune serum at identical dilution was used in panel C, and both panel C and D were processed for alkaline phosphatase detection which results in a blue-purple staining. Panels C and D were counter stained with eosin.

Fig 11 Schematic representation of the notch4, Notch4/int-3 and Jagged-1 proteins (A). Conserved domains within each proteins are indicated. Immunoblot analysis on lysates of 293 cells transiently transfected with either epitope tagged or non-epitope tagged cDNA's of Notch4/int or Notch4 (B and C) using anti-HA antibody (B) or anti-Notch4 antibody (C). Immunoblot analysis on lysates of RBE4 cells programmed to express LacZ, Notch4/int-3, Jagged-1 or Notch4 using the anti-HA antibody, demonstrates expression of each respective protein (D).

Fig 12 RBE4 cells that are programmed to express either LacZ (A) or Notch4 (B) display a cobble stone morphology when grown on collagen coated plates. RBE4 cells programmed to express Notch4/int-3 (C and E) or Jagged-1 (D and E) display a spindle shape morphology and spontaneously form microvessel structures. Structures were more

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prominent in RBE-Notch4/int-3 than in RBE-Jagged-1 cell cultures (compare E to F). Photographs A to D were taken at 100X magnification whereas photographs E and F were taken at 40X magnification.

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Fig 13 Histochemical analysis of alkaline phosphatase (B) and gamma-glutamyl transpeptidase[®] activities in microvessel structures induced by RBE4 cells expressing Notch4/int-3 (B) or Jagged-1 (C). RBE4 cells surrounding the microvessel structures do not express either enzyme activity (B and C). Panel A is control.

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Fig 14 Northern blot analysis on RBE4 cells. Panel A, 40 µg of total RNA (lane 2 and 4) from RBE4 cells programmed to express either LacZ, Notch4/int-3, Notch4 or Jagged-1, was hybridized to riboprobes for either Jagged-1, Notch1, Notch4 or β-actin. LTR-driven transcripts are denoted (LTR).

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Detailed Description of the Invention

This invention is illustrated in the Experimental Details section which follows. These sections are set forth to aid in an understanding of the invention but are not intended to, and should not be construed to, limit in any way the invention as set forth in the claims which follow thereafter.

Throughout this application, the following standard abbreviations are used to indicate specific nucleotide bases:

| | |
|------------|-----------|
| C=cytosine | A=adenine |
| T=thymine | G=guanine |

The activity of a protein such as a Notch4 protein may be measured using any of a variety of appropriate functional assays in which activation of the protein in question results in an observable change in the level of some second messenger system.

This invention provides an isolated nucleic acid encoding Notch4 protein.

This invention provides the Notch4 nucleic acid, wherein the nucleic acid is a DNA. In an embodiment, the Notch4 nucleic acid has the nucleic acid sequence as described in figure 9. In an embodiment, the DNA is a cDNA. In another embodiment, the DNA is a genomic DNA. In another embodiment the DNA is synthetic DNA. In still another embodiment, the nucleic acid is RNA. In a separate embodiment, the nucleic acid encodes Notch4 protein. As used herein, Notch4 protein has the amino acid sequence as described in Figure 1.

As used herein, Notch4 protein includes any polypeptide having Notch4 protein activity and having an amino acid

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sequence homologous to the amino acid sequence of Notch4. Thus, this term includes any such polypeptides whether naturally occurring and obtained by purification from natural sources or non-naturally occurring and obtained synthetically, e.g. by recombinant DNA procedures. Moreover, the term includes any such polypeptide where its sequence is substantially the same as, or identical to the sequence of any mammalian homolog of the human polypeptide, e.g. murine, bovine, porcine, etc. homologs. Additionally, the term includes mutants or other variants of any of the foregoing which retain at least some of the biological activity of nonmutants or nonvariants.

This invention also encompasses DNAs and cDNAs which encode amino acid sequences which differ from those of Notch4 protein, but which should not produce phenotypic changes. In addition, this invention also encompasses DNAs and cDNAs which hybridize to the DNA and cDNA of the subject invention. Hybridization methods are well known to those of skill in the art.

This invention provides an isolated nucleic acid encoding an activated Notch4 protein.

The nucleic acid of the subject invention also include DNA coding for polypeptide analogs, fragments or derivatives of polypeptides which differ from naturally-occurring forms in terms of the identity or location of one or more amino acid residues (deletion analogs containing less than all of the residues specified for the protein, substitution analogs wherein one or more residues specified are replaced by other residues and addition analogs where in one or more amino acid residues is added to a terminal or medial portion of the polypeptides) and which share some or all properties of naturally-occurring forms. These nucleic acids include: the incorporation of codons "preferred" for expression by selected non-mammalian hosts; the provision

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of sites for cleavage by restriction endonuclease enzymes;
and the provision of additional initial, terminal or
intermediate DNA sequences that facilitate construction of
readily expressed vectors.

5

The nucleic acids described and claimed herein are useful
for the information which they provide concerning the amino
acid sequence of the polypeptide and as products for the
large scale synthesis of the polypeptide by a variety of
recombinant techniques. The nucleic acid is useful for
generating new cloning and expression vectors, transformed
and transfected prokaryotic and eukaryotic host cells, and
new and useful methods for cultured growth of such host
cells capable of expression of the polypeptide and related
products.

15

This invention also provides an isolated Notch4 protein.
In one embodiment the Notch4 protein has the amino acid
sequence as shown in Figure 1.

20

This invention provides a vector comprising the above-
described nucleic acid.

Vectors which comprise the isolated nucleic acid described
hereinabove also are provided. Suitable vectors comprise,
but are not limited to, a plasmid or a virus. These
vectors may be transformed into a suitable host cell to
form a host cell vector system for the production of a
polypeptide having the biological activity of Notch4
protein.

30

This invention provides the above-described vector adapted
for expression in a cell which further comprises the
regulatory elements necessary for expression of the nucleic
acid in the cell operatively linked to the nucleic acid
encoding Notch4 protein as to permit expression thereof.

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This invention provides the above-described vector adapted for expression in a bacterial cell which further comprises the regulatory elements necessary for expression of the nucleic acid in the bacterial cell operatively linked to the nucleic acid encoding Notch4 protein so as to permit expression thereof.

This invention provides the above-described vector adapted for expression in a yeast cell which comprises the regulatory elements necessary for expression of the nucleic acid in the yeast cell operatively linked to the nucleic acid encoding Notch4 protein so as to permit expression thereof.

This invention provides the above-described vector adapted for expression in a plant cell which comprises the regulatory elements necessary for expression of the nucleic acid in the insect cell operatively linked to the nucleic acid encoding Notch4 protein so as to permit expression thereof.

In an embodiment, the vector is adapted for expression in a animal cell which comprises the regulatory elements necessary for expression of the DNA in the animal cell operatively linked to the DNA encoding animal Notch4 protein so as to permit expression thereof.

This invention provides a plasmid which comprises the regulatory elements necessary for expression of DNA in a cell operatively linked to the DNA encoding Notch4 protein as to permit expression thereof designated pBS-Notch4 (ATCC Accession No.).

This plasmid (pBS-Notch4) was deposited on June 12, 1997, with the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Maryland 20852, U.S.A. under the provisions of the Budapest Treaty for the International

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Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure and was accorded ATCC Accession No.

5 This invention provides a mammalian cell comprising the above-described plasmid or vector. In an embodiment, the mammalian cell is a Rat Brain Microvessel endothelial (RBE4) cell.

10 This invention provides a method of transforming host cells produced by transfecting host cells with the above-described vector.

15 This invention provides a nucleic acid probe comprising a nucleic acid molecule of at least 12 nucleotides capable of specifically hybridizing with a unique sequence included within the sequence of a nucleic acid molecule encoding Notch4 protein. In an embodiment, the nucleic acid is DNA.

20 The nucleic acid probe can either be DNA or RNA. As used herein, the phrase "specifically hybridizing" means the ability of a nucleic acid to recognize a nucleic acid sequence complementary to its own and to form double-helical segments through hydrogen bonding between
25 complementary base pairs.

This nucleic acid of at least 12 nucleotides capable of specifically hybridizing with a sequence of a nucleic acid encoding Notch4 can be used as a probe. Nucleic acid probe
30 technology is well known to those skilled in the art who will readily appreciate that such probes may vary greatly in length and may be labeled with a detectable label, such as a radioisotope or fluorescent dye, to facilitate detection of the probe. DNA probe molecules may be
35 produced by insertion of a DNA molecule which encodes Notch4 into suitable vectors, such as plasmids or bacteriophages, followed by transforming into suitable

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bacterial host cells, replication in the transformed bacterial host cells and harvesting of the DNA probes, using methods well known in the art. Alternatively, probes may be generated chemically from DNA synthesizers.

5

RNA probes may be generated by inserting the DNA which encodes Notch4 protein downstream of a bacteriophage promoter such as T3, T7 or SP6. Large amounts of RNA probe may be produced by incubating the labeled nucleotides with the linearized fragment where it contains an upstream promoter in the presence of the appropriate RNA polymerase.

10

This invention also provides a nucleic acid of at least 12 nucleotides capable of specifically hybridizing with a sequence of a nucleic acid which is complementary to the mammalian nucleic acid encoding Notch4 protein. This nucleic acid may either be a DNA or RNA molecule.

15

This invention provides an antisense oligonucleotide having a sequence capable of specifically hybridizing to mRNA encoding Notch4 protein so as to prevent translation of the mRNA.

20

This invention provides an antisense oligonucleotide having a sequence capable of specifically hybridizing to the genomic DNA of Notch4 protein or activated Notch4 protein.

25

This invention provides an antisense oligonucleotide of Notch4 comprising chemical analogues of nucleotides.

30

This invention provides an antibody directed to Notch4 protein. This invention also provides an antibody directed to activated Notch4 protein.

35

As used herein, the term "antibody" encompasses fragments of the antibody such that fragments are capable of binding to the specific antigen. Antibody also includes polypeptides

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which have the antigen binding domains. Such polypeptide may contain a single chain known as single-chain antibody. As used herein, peptide encompasses both polypeptide and oligopeptide.

5 Polyclonal antibodies against these peptides may be produced by immunizing animals using the selected peptides. Monoclonal antibodies are prepared using hybridoma technology by fusing antibody producing B cells from
10 immunized animals with myeloma cells and selecting the resulting hybridoma cell line producing the desired antibody. Alternatively, monoclonal antibodies may be produced by in vitro techniques known to a person of ordinary skill in the art. These antibodies are useful to
15 detect the expression of mammalian PSM antigen in living animals, in humans, or in biological tissues or fluids isolated from animals or humans.

This invention provides a monoclonal antibody directed to
20 an epitope of Notch4 present on the surface of a Notch4 receptor expressing cell.

This invention provides a pharmaceutical composition comprising an amount of the oligonucleotide effective to
25 reduce activity of Notch4 protein by passing through a cell membrane and binding specifically with mRNA encoding Notch4 in the cell so as to prevent its translation and a pharmaceutically acceptable carrier capable of passing through a cell membrane. In an embodiment, the
30 oligonucleotide is coupled to a substance which inactivates mRNA.

This invention provides the above-described pharmaceutical composition, wherein the pharmaceutically acceptable
35 carrier capable of passing through a cell membrane comprises a structure which binds to a receptor specific for a selected cell type and is thereby taken up by cells

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of the selected cell type.

5 This invention provides a pharmaceutical composition comprising an amount of an antagonist effective to reduce the activity of Notch4 protein and a pharmaceutically acceptable carrier.

10 This invention provides a pharmaceutical composition comprising an amount of an agonist effective to increase activity of Notch4 protein and a pharmaceutically acceptable carrier.

15 This invention provides the above-described pharmaceutical composition which comprises an amount of the antibody effective to block binding of a ligand to the Notch4 protein and a pharmaceutically acceptable carrier.

20 As used herein, "pharmaceutically acceptable carriers" means any of the standard pharmaceutically acceptable carriers. Examples include, but are not limited to, phosphate buffered saline, physiological saline, water and emulsions, such as oil/water emulsions.

25 This invention also provides a method for determining whether a ligand can specifically bind to Notch4 which comprises contacting a cell transfected with and expressing DNA encoding Notch4 with the ligand under conditions permitting binding of ligands to Notch4, detecting the presence of any such ligand specifically bound to Notch4,
30 and thereby determining whether the ligand specifically binds to Notch4.

35 This invention provides a method for determining whether a ligand can specifically bind to Notch4 protein comprising the steps of: a) contacting Notch4 protein with the ligand under conditions permitting formation of specific complexes between Notch4 protein and known Notch4 protein-

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binding ligands; b) determining whether complexes result from step (a), the presence of such complexes indicating that the ligand specifically binds to Notch4 protein.

- 5 In one embodiment, the Notch4 protein in step (a) described above, is reconstituted in liposomes. In another embodiment, the ligand is not previously known to be a ligand which can specifically bind to Notch4 protein.
- 10 This invention provides a method for determining whether a ligand can specifically bind to Notch4 which comprises contacting a cell transfected with and expressing DNA encoding Notch4 with the ligand under conditions permitting binding of ligands to Notch4, detecting the presence of any
- 15 such ligand specifically bound to Notch4, and thereby determining whether the ligand specifically binds to Notch4, such Notch4 having substantially the same amino acid sequence shown in Figure 1.
- 20 This invention provides a method for determining whether a ligand can specifically bind to Notch4 which comprises contacting a cell transfected with and expressing DNA encoding Notch4 with the ligand under conditions permitting binding of ligands to Notch4, detecting the presence of any
- 25 such ligand specifically bound to Notch4, and thereby determining whether the ligand specifically binds to Notch4, such Notch4 being characterized by an amino acid sequence in the transmembrane region having 60% homology or higher to the amino acid sequence in the transmembrane
- 30 region of Notch4 shown in Figure 1.

- This invention provides a method for determining whether a ligand can specifically bind to Notch4 which comprises preparing a cell extract from cells transfected with and
- 35 expressing DNA encoding Notch4, isolating a membrane fraction from the cell extract, contacting the membrane fraction with the ligand under conditions permitting

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binding of ligands to such protein, detecting the presence of the ligand specifically bound to Notch4, and thereby determining whether the ligand specifically binds to Notch4.

5

This invention provides a method for determining whether a ligand can specifically bind to Notch4 which comprises preparing a cell extract from cells transfected with and expressing DNA encoding Notch4, isolating a membrane
10 fraction from the cell extract, contacting the membrane fraction with the ligand under conditions permitting binding of ligands to Notch4, detecting the presence of the ligand specifically bound to Notch4, and thereby determining whether the ligand can specifically bind to
15 Notch4, such Notch4 having substantially the same amino acid sequence shown in Figure 1.

This invention provides a method for determining whether a ligand is a Notch4 protein agonist which comprises
20 contacting a cell transfected with and expressing Notch4 protein with the ligand under conditions permitting activation of a functional Notch4 protein response by ligands known to be agonists of Notch4 protein, and detecting whether a functional increase in Notch4 protein
25 activity occurs so as to determine whether the ligand is a Notch4 protein agonist.

The term "agonist" is used throughout this application to indicate any peptide or non-peptidyl compound which
30 increases the activity of any of the receptors/proteins of the subject invention.

This invention provides a method for determining whether a ligand is a Notch4 protein antagonist which comprises
35 contacting a cell transfected with and expressing Notch4 protein with the ligand under conditions permitting activation of a functional Notch4 protein response by

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ligands known to be agonists of Notch4 protein, and detecting whether a functional decrease in Notch4 protein activity occurs so as to determine whether the ligand is a Notch4 antagonist.

5

The term "antagonist" is used throughout this application to indicate any peptide or non-peptidyl compound which decreases the activity of any of the receptors/proteins of the subject invention.

10

This invention provides a pharmaceutical composition which comprises an amount of a Notch4 protein agonist effective to increase the activity of a Notch4 protein within a cell and a pharmaceutically acceptable carrier. In an embodiment, the pharmaceutical composition of comprises a peptide fragment of Jagged protein capable of increasing the activity of Notch4 protein.

15

This invention also provides a pharmaceutical composition which comprises an amount of a Notch4 protein antagonist effective to decrease activity of Notch4 protein within a cell and a pharmaceutically acceptable carrier.

20

This invention provides a method of modulating angiogenesis in a subject comprising administering to the subject an amount of agonist or antagonist of Notch4 protein effective to promote or inhibit angiogenesis in the subject.

25

This invention provides a method of promoting angiogenesis in a subject comprising administering to the subject an amount of Notch4 protein agonist effective to promote angiogenesis in the subject.

30

This invention provides a method of inhibiting angiogenesis in a subject comprising administering to the subject an amount of Notch4 protein antagonist effective to inhibit angiogenesis in the subject.

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This invention provides a method of promoting angiogenesis comprising transducing selected cells, wherein the cells express activated Notch4 protein in an amount sufficient to promote angiogenesis in the cells.

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This invention provides a method of promoting angiogenesis comprising transducing selected cells which express Notch4 protein, wherein the cells express a Notch4 ligand in an amount sufficient to promote angiogenesis in the cells.

10

This invention provides a method of promoting angiogenesis comprising transducing selected cells, wherein the cells express a Notch4 protein and a Notch4 protein ligand in an amount sufficient to promote angiogenesis in the cells. In an embodiment, the Notch4 protein ligand is Jagged protein or a peptide fragment thereof capable of increasing the activity of Notch4 protein.

15

This invention provides a method of inhibiting angiogenesis comprising administering to cells expressing Notch4 protein an amount of an antibody effective to block binding of a ligand to Notch4 protein.

20

This invention also provides a method of inhibiting angiogenesis comprising administering to cells expressing Notch4 protein an amount of a fragment of antibody effective to block binding of a ligand to Notch4 protein.

25

This invention provides a method of treating an abnormality in a subject wherein the abnormality is alleviated by inhibiting angiogenesis comprising blocking Notch4 protein signaling in the subject. In an embodiment, the above-described method comprises administering to the subject an amount of an antibody effective to block binding of a ligand to Notch4 protein. In another embodiment, the above-described method of treating an abnormality in a subject comprises administering to the subject an amount of

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a fragment of an antibody effective to block binding of a ligand to Notch4 protein. In another embodiment, the abnormality is a solid tumor, hemangioma, hemangiosarcoma or Kaposi's Sarcoma.

5

This invention provides a method of treating an abnormality in a subject wherein the abnormality is alleviated by promoting angiogenesis comprising stimulating the Notch4 signaling pathway. In an embodiment, the above-described method of treatment comprises transducing selected cells within the subject, wherein the cells express activated Notch4 protein. In another embodiment, the above-described method of treatment comprises transducing selected cells within the subject expressing a Notch4 protein such that the cells express a Notch4 protein ligand. In another embodiment, the above-described method of treatment comprises transducing selected cells within the subject such that the cells express a Notch4 protein and a Notch4 protein ligand. In an embodiment, the ligand is Jagged protein or a peptide fragment thereof capable of increasing the activity of Notch4 protein.

In an embodiment, the abnormality is an ischemic disorder, gangrene, diabetes ulceratis, chronic ulceration, Cerebral Autosomal Dominant Arteriopathy with Subcortical Infarcts and Leukoencephalopathy (CADASIL), Vascular Dementia or a wound.

This invention provides a method of detecting expression of Notch4 by detecting the presence of mRNA coding for Notch4 which comprises obtaining total mRNA from the cell and contacting the mRNA so obtained with the above-described nucleic acid probe under hybridizing conditions, detecting the presence of mRNA hybridized to the probe, and thereby detecting the expression of Notch4 by the cell.

This invention provides a method of detecting the presence

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of Notch4 on the surface of a cell which comprises
contacting the cell with the antibody capable of binding to
Notch4 under conditions permitting binding of the antibody
to the receptor, detecting the presence of the antibody
5 bound to the cell, and thereby detecting the presence of
Notch4 on the surface of the cell.

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Experimental Details

First Series of Experiments

Materials and Methods

5 Isolation and sequencing of Notch4 cDNA clones

A 1680 bp fragment was amplified by PCR from adult mouse testis cDNA (RT-PCR) using specific primers (5' primer: CGTCCTGCTGCGCTTCCTTGCA (Seq. I.D. No.) and 3' primer CCGGTGCCTAGTTTCAGATTTCTTA) (Seq. I.D. No.) designed from the int-3 cDNA sequence (Robbins et al., 1992). This cDNA fragment corresponds to the previously cloned int-3 oncogene. Two consecutive 5' RACE reactions (5'-Amplifinder RACE kit, Clontech®) using testis and lung cDNA were done to obtain cDNA clones located 5' of the int-3 oncogene. The above described cDNAs were cloned into Bluescript KS (Stratagene®) and the TA cloning vector (Invitrogen®) and used to generate probes to screen a lung cDNA library (Clontech®). Briefly, nitrocellulose membranes (Schleicher&Schuell) were hybridized in a solution containing 50% Formamide, 3x SSC, 100 mM Tris-HCl (pH 7.4), 5x Denhardt's solution, 0.2% SDS, and 0.1 mg/ml salmon sperm DNA at 42°C for 14 hours. Filters were then washed in 1x SSC and 0.5% SDS at room temperature followed by washes at 65°C. Positive clones were purified and sequenced to confirm overlapping regions. Novel 5' restriction fragments of these newly isolated clones were used in consecutive screens in order to obtain the full length Notch4 cDNA. All the above described clones were sequenced using the dideoxy termination method (Sanger) with an automatic DNA sequencer (Applied Biosystems®). Sequence data from both strands were obtained for the entire Notch4 cDNA and were analyzed and assembled using computer software (MacVector®).

35 Northern Blot analysis

Total RNA was isolated from adult CD-1 mouse tissues and Northern blot hybridization analysis was performed. 20 µg

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of total RNA was electrophoresed on a 1% agarose gel containing 6% formaldehyde. After electrophoresis RNAs were transferred to a nylon membrane (Duralon-UV membranes[®], Stratagene[®]) by capillary blotting. ³²P-radiolabeled riboprobes were transcribed (Maxiscript[®] in vitro transcription kit, Ambion[®]) from Notch4 cDNA clones encoding the 5' or 3' UTR (untranslated region) or ORF (open reading frame). The 3' UTR Notch4 cDNA clone was isolated by RT-PCR and a 440 bp restriction fragment of this cDNA was used as riboprobe. Hybridization solution contained 60% Formamide, 5x SSC, 5x Denhardt's solution, 1% SDS, 20 mM NaH₂PO₄ (pH 6.8), 0.1 mg/ml salmon sperm DNA, 100 ug/ml yeast tRNA, 10 ug/ml poly-A mRNA and 7% dextran sulfate and was done for 14 hours at 65°C. Washing solution contained 2x SSC and 1% SDS and was done at room temperature and 50°C for 15 minutes each, followed by a 2 hour wash at 80°C with a solution containing 0.2x SSC and 1% SDS. Membranes were exposed on X-ray film (X-OMAT AR, Kodak[®]). The integrity of the RNA, as well as comparable amounts of RNA were tested by rehybridization with a GAPDH probe.

In situ hybridization

Staged embryos ranging from 9 days to birth were obtained from timed breedings of CD-1 mice. Morning of the vaginal plugs was counted as 0.5 days post coitum (p.c.). Preparation of tissue and subsequent procedures for in situ hybridization were done as previously described (Marazzi and Buckley, 1993; Sassoon and Rosenthal, 1993). After hybridization and procedures, sections were dehydrated rapidly and processed for standard autoradiography using NTB-2 Kodak[®] emulsion and exposed for two weeks at 4°C. Analysis was carried out using both light and dark field optics on a Leica[®] DA microscope. To avoid potential cross-hybridization with homologous RNAs, an antisense ³⁵S-labeled RNA probe corresponding to the 3' UTR of Notch4 was used. Probes were used at a final concentration of 9x10⁴ dpm/ml.

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Results

Isolation and Analysis of Notch4 cDNA clones

The int-3 mammary oncogene encodes a truncated protein that is highly homologous to the intracellular part of the Notch receptor proteins. The full length int-3 gene, referred to as Notch4, had been proposed to encode a novel member of the Notch protein family (Robbins et al., 1992). To prove this hypothesis, cDNAs containing the complete coding potential of the Notch4 gene have been cloned. Using primers derived from the published sequence of the int-3 oncogene, RT-PCR was used to isolate a 2.4 kb int-3 cDNA encoding the putative intracellular portion of the receptor. To obtain cDNA clones encompassing the full coding potential of the normal int-3 gene, cDNAs were isolated by 5' RACE and by screening a mouse lung cDNA library. A total of 37 overlapping cDNA clones were analyzed and sequenced to obtain a 6677 bp cDNA sequence. This sequence encodes one long open reading frame of 1964 amino acids, starting with an initiator methionine at nucleotide 347 and terminating with a stop codon at nucleotide 6239. The 6677 bp cDNA corresponds in size to that of Notch4 transcripts detected by Northern blot analysis; thus, this suggests that the cloned cDNA represents the full length Notch4 gene.

Several differences (insertions, deletions, and single nucleotide changes) were found between the nucleotide sequence of Notch4 reported here and the previously published int-3 nucleotide sequence (Robbins et al., 1992). These differences alter the reading frame in several locations within the intracellular domain and may be a result of differences in sequence analysis or possibly mutations found in the tumor derived int-3 transcript (Robbins et al., 1992) that are not found in the Notch4 gene. The nucleotide sequence of mouse Notch4 has been deposited with Genbank under the Accession number U43691.

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Analysis of the Notch4 deduced amino acid sequence

Analysis of the deduced amino acid sequence of Notch4 reveals the presence of conserved domains shared by all Notch proteins (see Fig. 1). Notch4 contains EGF-like repeats, Notch/lin-12 repeats, a transmembrane domain, cdc10/ankyrin repeats and a putative PEST domain. The overall homology between Notch4 and other Notch proteins was determined using GCG (Bestfit, gap weight 3.0, length weight 0.1). The Notch4 protein is approximately 60% similar and 43% identical to other vertebrate Notch proteins, and 58% similar and 40% identical to Drosophila Notch. Lower homologies were found when compared to the C. elegans lin-12 and glp-1 proteins (49% similar and 29% identical).

Two hydrophobic regions in the Notch4 protein sequence were identified by hydropathy analysis (Kyte Doolittle algorithm, data not shown). An N-terminal region contains 19 hydrophobic residues that could function as a signal peptide sequence (Fig. 1) and a putative signal peptidase cleavage site was identified at residue 20. A second hydrophobic region from amino acid residues 1441 to 1465 is of sufficient length (25 amino acids) to behave as a membrane spanning domain and is immediately followed by five consecutive arginine residues that are consistent with a stop transfer signal (Fig. 1).

The extracellular domain of Notch4 contains 29 EGF-like repeats (Figs 1, 2), in contrast to the 36 EGF-like repeats found in murine Notch 1 (Franco Del Amo et al., 1993) and rat Notch 2 (Weinmaster et al., 1992), and to the 34 EGF-like repeats found in murine Notch 3 (Lardelli et al., 1994). EGF-like repeats are defined by a cysteine-rich consensus sequence and generally occur in analogous locations in two different Notch proteins. Since analogous repeats are more homologous to each other than to their neighboring EGF-like repeats, they have been referred to in

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Notch proteins as equivalent EGF-like repeats. The relationship between particular EGF-like repeats of other Notch proteins and those of the Notch4 protein was analyzed. Figure 2 schematizes the relationship of EGF-equivalents between Notch4 and Notch1/Notch2. EGF-like repeats 1-13 of Notch4 are equivalent to EGF-like repeats 1-13 of Notch1/Notch2, EGF-like repeats 22-24 of Notch4 correspond to EGF-like repeats 28-30 of Notch1/Notch2, and EGF-like repeats 26-29 of Notch4 are equivalent to EGF-like repeats 33-36 of Notch1/Notch2. Comparison of Notch4 to other Notch proteins revealed no clear-cut identification of the seven particular equivalent EGF-like repeats that are absent in Notch4. The amino acid sequence of equivalent EGF-like repeats has diverged between different Notch homologues and orthologues (Maine et al., 1995), sometimes resulting in loss of a clear-cut equivalent repeat consensus. Six of the unassigned EGF-like repeats of Notch4 appear to be derived from EGF-like repeats 14-27 of Notch1 and Notch2 (Fig. 2). EGF-like repeat 25 of Notch4 may be a hybrid EGF-like repeats derived from parts of EGF-like repeats 31 and 32 of Notch1/Notch2 (Fig. 2). A discussion of the relationship between Notch3 and Notch1/Notch2 (shown in Fig. 2) is described in Lardelli et al., 1994.

EGF-like repeats 11 and 12 of Drosophila Notch have been shown to be necessary and sufficient for Notch to bind Delta and Serrate proteins in vitro (Rebay et al., 1991). These two equivalent EGF-like repeats are present in Notch4 (Fig 2). The putative calcium-binding residues (Handford et al., 1991) in EGF-like repeat 11 are also conserved in Notch4 (Fig. 3). The residues between the first and second cysteine of EGF-like repeat 11 have been shown in *Xenopus* Notch to be important in ligand binding, and are divergent between Notch proteins (Fig. 3). In this region, Notch4 has additional residues and is unique when compared to other murine Notch proteins.

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Notch4 also contains three Notch/lin-12 repeats which are approximately 53% identical to the Notch/lin-12 repeats found in other murine Notch proteins. Between the Notch/lin-12 repeats and the transmembrane domain of Notch4
5 are 2 cysteines at positions 1388 and 1397 that are conserved among all Notch proteins and may promote receptor dimerization upon ligand binding (Greenwald and Seydoux, 1990).

10 The intracellular domain of Notch4 contains the 6 ankyrin/cdc10 repeats found in other Notch proteins. The ankyrin repeat domain of Notch4 is 48%, 52%, and 55% identical to the ankyrin repeat domains of Notch1, Notch2, and Notch3 respectively. In all Notch proteins the number
15 of amino acids between the transmembrane domain and the ankyrin/cdc10 repeats is 110 residues, as it is in Notch4 (Fig. 1). Like other Notch proteins, Notch4 contains a C-terminal PEST domain, albeit of shorter length. In addition, Notch4 lacks a recognizable opa repeat (Fig. 1),
20 such as that found in Drosophila Notch. The carboxy-terminal end of Notch proteins, beyond the ankyrin/cdc10 repeats, is the least conserved region among Notch proteins. Within this C-terminal region, Notch4 displays little homology to other Notch proteins and no significant
25 homology to other known proteins. This C-terminal is also much shorter in Notch4 than in other Notch proteins, containing 177 residues, compared to 457 in Notch1, 437 in Notch2, and 329 in Notch3.

30 Expression analysis of Notch4 in adult tissues

Several adult tissues were examined for the presence of Notch4 transcripts by Northern blot analysis. To minimize cross-hybridization with other mouse Notch transcripts, a riboprobe derived from the 3' UTR of Notch4 was used. In
35 most tissues analyzed, a single hybridizing species of 6.7 kb was detected (Fig. 4), which roughly corresponds in size to the cloned Notch4 cDNA. The 6.7 kb transcript is most

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highly expressed in lung, at lower levels in heart and kidney, and at detectable levels in ovary and skeletal muscle. Very low levels of the 6.7 kb transcript were observed in several other adult tissues; including brain,
5 intestine, liver, testis (Fig. 4) and spleen (data not shown). In adult testis, two abundant transcripts of 1.5 kb and 1.1 kb were observed. Thus, Notch4 expression varies widely in adult tissues. Other than in testis, transcript size variation in different tissues was not
10 detected.

Analysis of testis-specific truncated Notch4 transcripts

To determine the cell lineage specificity of Notch4 expression in the murine testis, RNA was analyzed in the
15 germ cell deficient mouse testis (Fig. 5). Mice that carry two mutations at the white-spotting locus (W/W^y) are devoid of germ cells, but have the normal complement of somatic cell types, including Leydig, Sertoli, and peritubular myoid cells (Mintz and Russell, 1957). Heterozygous litter
20 mates (W/+) have normal somatic and germ cell complements. Northern blot analysis of total RNA from germ cell-deficient testes (W/W^y) and testes with normal germ cells (W/+ and adult (+/+)) was done using a riboprobe derived from the 3' UTR (probe D in Fig. 5C). Transcripts of 1.5 kb
25 and 1.1 kb were detected in RNA from the testes of adult wild-type mice and W/+ mice (Fig. 5A). However, neither transcript was detected in RNA from homozygous mutant testes, suggesting that these transcripts were likely to be specific to the germinal compartment.

30 Since spermatogenic differentiation undergoes a characteristic temporal progression, one can use mice testes at specific days of postnatal development to enrich for or eliminate particular germ cell types. Testes from
35 day 7 of postnatal development (day 7 p.n.) mice contain mitotic spermatogonia while testes from day 17 p.n. mice have entered meiosis and have progressed to spermatocytes

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(Nebel et al., 1961). Both day 7 p.n. and day 17 p.n. testes lack post-meiotic spermatids. Total RNA from immature and adult testes was analyzed by Northern blot hybridization to determine stage-specific expression of Notch4 transcripts during male germ cell development. Both Notch4 transcripts of 1.5 kb and 1.1 kb are absent in day 7 p.n. and day 17 p.n. testis, but are present in adult testis (Fig. 5A). These results indicate that the expression of the 1.5 kb and 1.1 kb Notch4 transcripts are restricted to post-meiotic germ cells.

To determine the nature of the short Notch4 transcripts in adult mouse testis, Northern blot analysis was done using riboprobes derived from different regions of the Notch4 coding sequence, as well as from 5' and 3' UTR (Fig. 5B). A riboprobe derived from the 5' UTR (probe A in Fig. 5C) failed to hybridize to either the 1.5 kb or the 1.1 kb transcripts (Fig. 5B); whereas this probe did hybridize to the 6.7 kb transcript found in lung RNA (Fig. 5B). However, riboprobes derived from the 3' UTR (probe D in Fig. 5C) or from cDNA encoding part of the intracellular domain of Notch4 (probe C in Fig. 5C) hybridize to the testis transcripts (Fig. 5A and data not shown). Probes derived from the coding sequence of the extracellular domain of Notch4 (probe B in Fig. 5C) did not hybridize to the testes transcripts (data not shown). To characterize the transcripts expressed in the adult mouse testis, a cDNA library prepared from adult mouse testes RNA was screened using probe C of Figure 5C. All the clones analyzed encoded the most C-terminal coding sequence and the 3' untranslated region of Notch4. Two independent clones of distinct size contained novel 5' sequences unrelated to that found in the full-length Notch4 cDNA (schematized in Fig 5C-Notch4 testis transcripts). Based upon the Northern blot analysis described above and the sequence of the cloned testis cDNAs, it is suggested here that Notch4 transcripts are either derived from an alternate intronic

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promoter that is active in post-meiotic germ cells or they may be driven by the same promoter as the 6.7 kb transcript and consist of spliced products derived from a 5' untranslated region upstream of what has currently been identified. The predicted amino acid sequence of the testis Notch4 transcripts with novel 5' sequence does not contain a methionine that could function as a translation initiator; therefore, these transcripts are unlikely to encode for protein products. The testis transcripts may thus represent aberrant transcriptional events in post-meiotic germ cells, as has been described previously (Davies and Willison, 1993).

Expression analysis of Notch4 during development

A 6.7 kb Notch4 transcript was detected by Northern hybridization in RNA isolated from day 12.5 p.c. mouse embryos (data not shown and Sarkar et al., 1994). To determine the spatial and temporal pattern of Notch4 transcript accumulation during development, mouse embryo tissue sections from 9.0 days p.c. to birth was examined using in situ hybridization. Throughout embryonic development, as well as in postnatal tissues (data not shown), Notch4 is highly expressed in endothelial cells. Intense labeling for Notch4 is observed in embryonic blood vessels at 9.0 days p.c. (Fig. 6A, B). As shown in Fig. 6 (panel C and D) strong labeling is observed over the dorsal aorta, the aortic tract, and the pulmonary artery in a 13.5 days p.c. embryo, while no labeling is detected in the epithelial cells lining the gut (red arrow). At higher magnification, it is noted that labeling is restricted to the endothelial cells lining the embryonic vessels (Fig. 6 D, E) and no labeling is detected in the red blood cells in the vessel. A weak and transient signal is also detectable in the developing nervous system from 9.0 days p.c. embryos. As shown in Figure 6 (panel A and B), a light diffuse labeling is detected in the developing nervous system, and a more distinct signal is observed at the tip

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of the neural folds. Notch4 transcripts in the nervous system are still detectable at 11.5 days p.c., but by 13.5 days p.c. no labeling for Notch4 is detectable in the nervous system (data not shown). In situ hybridization on adult lung and kidney sections was performed to determine the cell types responsible for Notch4 expression (data not shown) and Notch4 transcripts were solely found in the endothelial compartment of these tissues. The endothelial specific expression likely underlies the abundance of Notch4 transcripts found by Northern blot analysis of highly vascularized adult tissues (lung, heart, and kidney in Fig. 4).

Discussion

It is reported here the identification of a novel mouse gene that exhibits structural homology with the vertebrate Notch protein family. This gene has been named Notch4, as it is the fourth murine Notch gene identified. Notch4 contains all the conserved domains characteristic of Notch proteins (Figs 1 and 2). However, Notch4 contains only 29 EGF-like repeats within its extracellular domain as compared to the 36 repeats found in Notch1 and Notch2. In addition, the C-terminal tail of Notch4, beyond the ankyrin/cdc10 repeats, is shorter and unique when compared to all other Notch proteins, however, little is known of the function of this region in Notch proteins. Notch4 also contains a distinct EGF-like repeat 11 which has been proposed to be crucial for ligand binding. Structural variation in this repeat, and differences in the number of EGF-like repeats between murine Notch proteins, may be important for ligand specificity among the different possible Notch ligands. It must be noted that Notch/lin-12 proteins of varying structure have been demonstrated to be functionally interchangeable; C. elegans glp-1 can fully substitute for lin-12 (Fitzgerald et al., 1993) for instance. Therefore, Notch4 may be functionally interchangeable with other murine Notch proteins despite

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structural differences between these Notch proteins.

Notch4 is distinct from other Notch family proteins based on its expression pattern during embryonic development and in the adult mouse. In situ hybridization demonstrates endothelial-specific embryonic expression of Notch4. This endothelial-specific expression of Notch4 remains in the adult mouse. A weak and transient labeling is seen in the neural tube between day 9 p.c. and 11.5 p.c., with a more intense labeling at the tips of neural folds. This region of the neural tube is a highly plastic area where cells will probably participate in the fusion process of the neural tube and/or migrate as neural crest. The Notch4 expression pattern is in sharp contrast to the expression patterns of Notch1, 2, and 3. These Notch genes are expressed in a variety of different embryonic tissues such as the developing brain and spinal cord, presomitic and somitic mesoderm, and a variety of epithelial cells and mesenchymal derived tissues (Weinmaster et al., 1991; Williams et al., 1995). Notch1 is the only other Notch gene reported to be expressed in endothelial cells (Reaume et al., 1992). Endothelial expression has not been reported for Jagged and Dll-1, two putative mammalian Notch ligands (Bettenhausen et al., 1995; Lindsay et al., 1995). Expression of Notch1 and 4 in endothelial cells might reflect either redundancy of function or distinct biological functions in endothelial development.

Since Notch proteins have been implicated in binary cell fate specification regulating how equivalent cells can give rise to cells with different fates, a putative biological function of Notch4 might be to govern the cell fate decisions during endothelial growth and development. In amniotes, endothelial and hematopoietic cells appear synchronously in the blood islands. In zebra fish, lineage data have shown that individual cells of the early blastula can give rise to both endothelial and blood cells,

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suggesting a common embryonic precursor which has been referred to as the "hemangioblast." The occurrence of binary cell fate decision events in the hemangioblast is supported by analysis of the endothelial and/or hematopoietic cell lineages. Cloche, bloodless, and spadetail are mutants isolated in zebra fish that display phenotypes defective in either the hematopoietic development or both hematopoietic and endothelial development (Stainier et al., 1995). In the mouse, the Flk-1 and the Flt-1 genes encode receptor tyrosine kinases that are expressed in embryonic endothelium (Shalaby et al., 1995, Fong et al., 1995). Null mutants for the Flk-1 gene are defective in endothelial and blood cell development (Shalaby et al., 1995), whereas null mutants for the Flt-1 gene display only hematopoietic cell development defects (Fong et al., 1995). Mutational analysis of the Notch4 gene in whole animals would help to define the role of Notch4 in endothelial cell growth and development.

Alterations in stem cell fate decisions as a result of activated Notch proteins have been proposed to contribute to mitogenic growth of tumor cells. Blocked cell differentiation of fated daughter cells by activated Notch proteins may lead to an increase in the number of cells undergoing cell division or a prolonged life of the cell. In these cells, the probability of secondary oncogenic mutations that contribute to neoplastic transformation would be enhanced. Although little is known about the nature of stem cells in the mammary epithelium, Notch4 might regulate the fate decisions of mammary epithelial cells. This hypothetical model may explain the phenotype that is observed in int-3 transgenic mice, which display blocked development of the mammary gland and develop mammary carcinomas at high frequency.

The signal transduction pathways by which Notch proteins

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function are becoming understood through genetic studies in Drosophila. Deltex and Suppressor of Hairless (Su(H)) have been demonstrated to bind to the cdc10 repeats of the intracellular domain of Drosophila Notch (Diederich et al., 1994; Fortini and Artavanis-Tsakonas, 1994; Matsuno et al., 1995). More recently the mammalian Su(H) orthologue RBP-Jk, a transcription factor, has been shown to bind to the intracellular domain of Notch 1 (Jarriault et al., 1995). Since Notch4 contains the canonical ankyrin/cdc10 repeats, RBP-Jk or RBP-Jk homologues and mammalian Deltex homologues may interact with the cdc10/ankyrin repeats of Notch4. It has been proposed that upon activation of the Notch receptors, Su(H) or RBP-Jk are activated and translocate to the nucleus where they may regulate transcription of target genes (Goodbourn, 1995). In fact, activated Notch proteins encoding the intracellular domain have been reported to localize to the nucleus (Kopan et al., 1994; Struhl et al., 1993) suggesting a nuclear function for this domain. It was found that an epitope-tagged version of the int-3 oncoprotein is also localized to the nucleus when expressed in cultured 293T cells, as determined by immunofluorescence (unpublished data). This finding may indicate that int-3 can bind to cytoplasmic proteins that are then translocated to the nucleus.

It is shown here that the int-3 gene encodes a truncated Notch4 protein with the extracellular domain deleted (EGF-like repeats and Notch/lin-12 repeats), providing the first comparison of a naturally-activated murine Notch protein and its normal counterpart. In MMTV-induced mouse mammary tumors with an activated Notch4, as described in Robbins et al. (1992), the oncogenic effects are likely the result of both over expression or ectopic expression of Notch4 mRNA as well as functional activation of the Notch4 protein. A structural comparison of the mutant int-3 protein to the normal Notch4 protein is reminiscent of the structural

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alterations reported to activate the effector function of
Drosophila Notch and C. elegans lin-12 proteins
(Greenwald, 1994) or oncogenic activation of TAN-1.
Thus, loss of the extracellular domain likely leads loss
5 of the regulatory controls provided by the ligand binding
domain believed to reside in the EGF-like repeats of
Notch4.

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References Corresponding to First Series of Experiments

- Artavanis-Tsakonas, S. and Simpson, P. (1991). Choosing a cell fate: a view from the Notch locus. [Review].
5 Trends in Genetics 7, 403-408.
- Artavanis-Tsakonas, S., Matsuno, K. and Fortini, M. E. (1995). Notch signaling. [Review]. Science 268, 225-232.
- 10 Bettenhausen, B., Hrabe de Angelis, M., Simon, D., Guenet, J.-L. and Gossler, A. (1995). Transient and restricted expression during mouse embryogenesis of Dll, a murine gene closely related to Drosophila Delta. Development 121, 2407-2418.
- 15 Coffman, C. R., Skoglund, P., Harris, W. A. and Kintner, C. R. (1993). Expression of an extracellular deletion of Xotch diverts cell fate in Xenopus Embryos. Cell 73, 659-671.
- 20 Conlòn, R. A., Reaume, A. G. and Reascend, J. (1995). Notch1 is required for the coordinate segmentation of somites. Development 121, 1533-1545.
- 25 Davies, O. P. and Willison, K. R. (1993). Molecular mechanisms of differentiation in mammalian spermatogenesis. Developmental Biology 3, 179-188.
- 30 Diederich, R. J., Matsuno, K., Hing, H. and Artavanis-Tsakonas, S. (1994). Cytosolic interaction between deltex and Notch ankyrin repeats implicates deltex in the Notch signaling pathway. Development 120, 473-481.
- 35 Ellisen, L. W., Bird, J., West, D. C., Soreng, A. L., Reynolds, T. C., Smith, S. D. and Sklar, J. (1991). TAN-1, the human homolog of the Drosophila notch gene, is broken by chromosomal translocations in T lymphoblastic

-47-

neoplasms. Cell 66, 649-661.

5 Fitzgerald, K., Wilkinson, H. A. and Greenwald, I. (1993).
glp-1 can substitute for lin-12 in specifying cell fate
decisions in *Caenorhabditis elegans*. Development 119,
1019-1027.

10 Fleming, R. J., Scottgale, T. N., Diederich, R. J. and
Artavanis-Tsakonas, S. (1990). The gene *Serrate* encodes
a putative EGF-like transmembrane protein essential for
proper ectodermal development in *Drosophila melanogaster*.
Genes & Development 4, 2188-2201.

15 Fong, G., Reascend, J., Gertsenstein, M. and Breitman, M.
L. (1995). Role of the Flt-1 receptor tyrosine kinase in
regulating the assembly of vascular endothelium. Nature
376, 66-70.

20 Fortini, M. E., Rebay, I., Caron, L. A. and Artavanis-
Tsakonas, S. (1993). An activated Notch receptor blocks
cell-fate commitment in the developing *Drosophila* eye.
Nature 365, 555-557.

25 Fortini, M. E. and Artavanis-Tsakonas, S. (1994). The
suppressor of hairless protein participates in notch
receptor signaling. Cell 79, 273-282.

30 Franco Del Amo, F., Gendron-Maguire, M., Swiatek, P. J.,
Jenkins, N. A., Copeland, N. G. and Gridley, T. (1993).
Cloning, analysis, and chromosomal localization of Notch-1,
a Mouse homolog of Drosophila Notch. Genomics 15, 259-264.

35 Gallahan, D. and Callahan, R. (1987). Mammary
tumorigenesis in feral mice: identification of a new int
locus in mouse mammary tumor virus (Czech II)-induced
mammary tumors. J. Virol. 61, 66-74.

-48-

- Goodbourn, S. (1995). Notch takes a short cut. Nature 377, 288-289.
- 5 Greenwald, I. (1985). lin-12, a nematode homeotic gene, is homologues to a set of mammalian proteins that includes epidermal growth factor. Cell 43, 583-590.
- 10 Greenwald, I. and Seydoux, G. (1990). Analysis of gain-of-function mutations of the lin-12 gene of *Caenorhabditis elegans*. Nature 346, 197-199.
- 15 Greenwald, I. and Rubin, G. M. (1992). Making a difference: the role of cell-cell interactions in establishing separate identities for equivalent cells. [Review]. Cell 68, 271-281.
- 20 Greenwald, I. (1994). Structure/function studies of lin-12/Notch proteins. [Review]. Current Opinion in Genetics & Development 4, 556-562.
- 25 Handford, P. A., Mayhew, M., Baron, M., Winship, P. R., Campbell, I. D. and Brownlee, G. G. (1991). Key residues involved in calcium-binding motifs in EGF-like repeats. Nature 351, 164-167.
- 30 Henderson, S. T., Gao, D., Lambie, E. J. and Kimble, J. (1994). lag-2 may encode a signaling ligand for the GLP-1 and LIN-12 receptors of *C. elegans*. Development 120, 2913-2924.
- 35 Jarriault, S., Brou, C., Logeat, F., Schroeter, E. H., Kopan, R. and Israel, A. (1995). Signaling downstream of activated mammalian Notch. Nature 377, 355-358.
- 40 Jhappan, C., Gallahan, D., Stahle, C., Chu, E., Smith, G. H., Merline, G. and Callahan, R. (1992). Expression of an activated Notch-related int-3 transgene interferes with

-49-

cell differentiation and induces neoplastic transformation in mammary and salivary glands. Genes Devel. 6, 345-355.

5 Kopan, R., Nye, J. S. and Weintraub, H. (1994). The intracellular domain of mouse Notch: a constitutively activated repressor of myogenesis directed at the basic helix-loop-helix region of MyoD. Development 120, 2385-2396.

10 Lardelli, M. and Lendahl, U. (1993). Motch A and Motch B-two mouse Notch homologues coexpressed in a wide variety of tissues. Exp. Cell Res. 204, 364-372.

15 Lardelli, M., Dahlstrand, J. and Lendahl, U. (1994). The novel Notch homologue mouse Notch3 lacks specific epidermal growth factor-repeats and is expressed in proliferating neuroepithelium. Mechanism of Development 46, 123-136.

20 Lindsell, C. E., Shawber, C. J., Boulter, J. and Weinmaster, G. (1995). Jagged: a mammalian ligand that activates Notch1. Cell 80, 909-917.

25 Maine, E. M., Lissemore, J. L. and Starmer, W. T. (1995). A phylogenetic analysis of vertebrate and invertebrate Notch-related genes. Molecular Phylogenetics and Evolution 4, 139-149.

30 Marazzi, G. and Buckley, K. M. (1993). Accumulation of mRNAs encoding synaptic vesicle-specific proteins precedes neurite extension during early neuronal development. Developmental Dynamics 197, 115-124.

35 Matsuno, K., Diederich, R. J., Go, M. J., Blaumeueller, C. M. and Artavanis-Tsakonas, S. (1995). Deltex acts as a positive regulator of Notch signaling through interactions with the Notch ankyrin repeats. Development 121, 2633-2644.

-50-

- Mello, C. C., Draper, B. W. and Priess, J. R. (1994). The maternal genes *apx-1* and *glp-1* and establishment of dorsal-ventral polarity in the early *C. elegans* embryo. Cell 77, 95-106.
- 5 Michaely, P. and Bennett, V. (1992). The ANK repeat: a ubiquitous motif involved in macromolecular recognition. Trends Cell Biol. 2, 127-129.
- 10 Mintz, B. and Russell, E. S. (1957). Gene-induced embryological modifications of primordial germ cell in the mouse. J. Exp. Zool. 134, 207-230.
- 15 Nebel, B. R., Amarose, A. P. and Hackett, E. M. (1961). Calendar of gametogenic development in the prepuberal male mouse. Science 134, 832-833.
- 20 Reaume, A. G., Conlon, R. A., Zirngibl, R., Yamaguchi, T. P. and Reascend, J. (1992). Expression analysis of a Notch homologue in the mouse embryo. J. Dev. Biol. 154, 377-387.
- 25 Rebay, I., Fleming, R. J., Fehon, R. G., Cherbas, L., Cherbas, P. and Artavanis-Tsakonas, S. (1991). Specific EGF repeats of Notch mediate interactions with Delta and Serrate: implications for Notch as a multi-functional receptor. Cell 67, 687-699.
- 30 Rebay, I., Fehon, R. G. and Artavanis-Tsakonas, S. (1993). Specific truncations of *Drosophila* Notch define dominant activated and dominant negative forms of the receptor. Cell 74, 319-329.
- 35 Robbins, J., Blondel, B. J., Gallahan, D. and Callahan, R. (1992). Mouse mammary tumor gene int-3, a member of the notch gene family transforms mammary epithelial cells. J. Virol. 66, 2594-2599.

-51-

- Rogers, S., Wells, R. and Rechsteiner, M. (1986). Amino acid sequences common to rapidly degrade proteins: The PEST hypothesis. Science **234**, 364-368.
- 5 Sarkar, N. H., Haga, S., Lehner, A. F., Zhao, W., Imai, S. and Moriwaki, K. (1994). Insertional mutation of int protooncogenes in the mammary tumors of a new strain of mice derived from the wild in China: normal- and tumor-tissue-specific expression of int-3 transcripts. Virology
10 **203**, 52-62.
- Sassoon, D. and Rosenthal, N. (1993). Detection of messenger RNA by in situ hybridization. Methods Enzymol.
15 Wassereman, P.M. and DePamphilis, M.L. (eds) V225, 384-404.
- Shalaby, F., Reascend, J., Yamaguchi, T. P., Gertsenstein, M., Wu, X., Breitman, M. L. and Schuh, A. C. (1995). Failure of blood-island formation and vasculogenesis in FLK-1-deficient mice. Nature **376**, 62-66.
- 20 Smith, G. H., Gallahan, D., Diella, F., Jhappan, C., Merlino, G. and Callahan, R. (1995). Constitutive expression of a truncated INT-3 gene in mouse mammary epithelium impairs differentiation and functional
25 development. Cell Growth & Differentiation **6**, 563-577.
- Stainier, D. Y. R., Weinstein, B. W., Detrich, H. W., Zon, L. I. and Fishman, M. C. (1995). cloche, an early acting zebra fish gene, is required by both the endothelial and
30 hematopoietic lineages. Development **121**, 3141-3150.
- Stifani, S., Blaumueller, C. M., Redhead, N. J., Hill, R. E. and Artavanis-Tsakonas, S. (1992). Human homologs of a Drosophila Enhancer of split gene product define a novel
35 family of nuclear proteins [published erratum appears in Nat Genet 1992 Dec;2(4):343]. Nature Genetics **2**, 119-127.

-52-

- Struhl, G., Fitzgerald, K. and Greenwald, I. (1993). Intrinsic activity of the Lin-12 and Notch intracellular domains in vivo. Cell 74, 331-345.
- 5 Sugaya, K., Fukagawa, T., Matsumoto, K., Mita, K., Takahashi, E., Ando, A., Inoko, H. and Ikemura, T. (1994). Three genes in the human MHC class III region near the junction with the class II: gene for receptor of advanced glycosylation end products, PBX2 homeobox gene and a notch
10 homolog, human counterpart of mouse mammary tumor gene int-3. Genomics 23, 408-419.
- Swiatek, P. J., Lindsell, C. E., Franco del Amo, F., Weinmaster, G. and Gridley, T. (1994). Notch 1 is
15 essential for postimplantation development in mice. Genes & Development 8, 707-719.
- Tax, F. E., Yeagers, J. J. and Thomas, J. H. (1994). Sequence of C. elegans lag-2 reveals a cell-signaling
20 domain shared with Delta and Serrate of Drosophila. Nature 368, 150-154.
- Vassin, H., Bremer, K. A., Knust, E. and Campos-Ortega, J. A. (1987). The neurogenic gene Delta of Drosophila
25 melanogaster is expressed in neurogenic territories and encodes for a putative transmembrane protein with EGF-like repeats. EMBO J. 6, 3431-3440.
- Weinmaster, G., Roberts, V. J. and Lemke, G. A. (1991).
30 A homolog of Drosophila Notch expressed during mammalian development. Development 113, 199-205.
- Weinmaster, G., Roberts, V. J. and Lemke, G. (1992).
Notch 2: a second mammalian Notch gene. Development 116,
35 931-941.
- Williams, R., Lendahl, U. and Lardelli, M. (1995).

-53-

Complementary and combinatorial patterns of Notch gene family expression during early mouse development. Mechanisms of Development 53, 357-368.

- 5 Yochem, J. and Greenwald, I. (1989). glp-1 and lin-12, genes implicated in distinct cell-cell interactions in *C. elegans*, encode similar transmembrane proteins. Cell 58, 553-563.

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Second Series of Experiments

Material and Methods

5 Reagents

Recombinant human basic fibroblast growth factor (bFGF) was kindly provided by Dr. Anthony Belve (Columbia University, New York). Rat tail collagen solution was obtained from Upstate Biotechnology Inc. (Lake Placid, NY). Anti-HA
10 monoclonal antibody (12CA5) was obtained from Berkeley Antibody Co. (Richmond, CA) and horseradish peroxidase-conjugated sheep anti-mouse immunoglobulin G from Amersham (Arlington Heights, IL). All restriction and DNA modifying enzymes were purchased from New England Biolab Inc.
15 (Beverly, MA).

cDNA's

The murine int-3 cDNA corresponds to a truncated Notch4 cDNA as described previously. The int-3 nucleotide
20 sequence corresponds to nucleotide 4551 to nucleotide 6244 of Notch4. An oligonucleotide sequence encoding the haemagglutinin (HA) antigenic determinant was appended to the 3' end of the int-3 and Notch4 cDNA's. These eighteen codons specified the amino acid sequence SMAYPYDVDPDYASLGPGP
25 (Sequence I.D. No.), including the nine amino acid-long HA epitope, as underlined. HA-tagged int-3 and Notch4 cDNAs were created by subcloning each cDNA into Bluescript KS (Stratagene®) that contained the coding region of the HA epitope situated downstream of the newly inserted cDNA,
30 separated from it by polylinker sequence. These two sequences were made co-linear by the "loop-out" mutagenesis procedure using oligonucleotides designed to eliminate the stop codon and non-coding 3' sequence of the int-3 and Notch4 cDNAs and flanking polylinker sequence. The
35 sequence of the oligonucleotides used to loop out int-3 and Notch4 specific sequences are CGG TTG TAA GAA ATC TGA ACT CCA TGG CCT ACC CAT ATG (Sequence I.D. No.). The 5' end of

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each oligonucleotide is complimentary to the C-terminus of int-3 or Notch4 cDNA and their 3' ends anneal to the beginning of the HA epitope-encoding sequence (underlined in oligonucleotide). Mutagenesis was accomplished using the Muta-Gene phagemid in vitro mutagenesis kit (Bio Rad, Richmond, CA). The presence of each fusion was confirmed by DNA sequencing. The JaggedHA cDNA, HA-tagged at the carboxy terminus, was obtained from Dr. Gerry Weinmaster (UCSF, California).

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Cell cultures

Rat Brain Microvessel endothelial cells (RBE4) cells were obtained from Dr. Francoise Roux (INSERM U26, Hopital F. Widal, Paris) and were routinely plated on collagen coated dishes and maintained in Alpha MEM/Ham's F10 (1:1) supplemented with 2mM glutamine, 10% fetal calf serum, 1ng/ml basic fibroblast growth factor (bFGF), and penicilline/streptomycin in humidified 5% CO2/95% air at 37C. Medium was changed twice a week, and cells were passaged at a split ratio of 1:4.

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Cell lines

HA-tagged cDNAs (int-3HA, JaggedHA, and Notch4HA) were inserted into the retroviral vector pLHTCX wherein hygromycin-resistance/thymidine kinase fusion gene phosphotransferase gene expression is controlled by the murine leukemia virus (MLV) long terminal repeat (LTR), and cDNA transcription is controlled by an internal cytomegalovirus (CMV) enhancer/promoter. Distinct populations of RBE4 cells, each expressing an HA-tagged int3 or Jagged cDNA, were prepared by retroviral infection. Recombinant retroviruses were generated by transiently transfecting pLHTCX constructs into the BOSC 23 packaging cell line by calcium phosphate co-precipitation. Retroviral infection of RBE4 cells was accomplished by culturing these cells in the presence of viral supernatants obtained from the transfected packaging cells two day post-

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transfection. Infections were carried out in the presence of 4 $\mu\text{g/ml}$ polybrene for 12 hours after which the medium was replaced to regular medium. One day post-infection the culture medium was replaced to regular medium containing 100 $\mu\text{g/ml}$ hygromycin B (Sigma Chemical Co.). Colonies appeared 5 days later and were pooled into medium containing 100 $\mu\text{g/ml}$ hygromycin B. These resultant populations, each comprised of at least 50 clones, were used in cellular and biochemical assays described below. Distinct populations of RBE4 cells expressing an HA-tagged Notch4 cDNA were prepared by calcium phosphate transfection directly into the RBE4 cells. Selection and pooling of resistant colonies was done as described above.

15 Immunoblot analysis

HA epitope tagged int-3, Jagged and Notch4 proteins from lysates of RBE4 cell populations were analyzed by immunoblotting. To maximize protein expression, RBE4 cells were treated with 2mM sodium butyrate for 16 hours prior to lysis. Cells were washed twice with cold PBS and, subsequently, removed from the dish in 1.5 ml PBS using a rubber policeman. Cells were pelleted by centrifugation at 2,000x g at 4°C for 5 min. and lysed in 90 μl TENT buffer (20 mM Tris, pH 8.0, 2 mM EDTA, 150 mM NaCl, 1% Triton-X100) containing 1 mM phenylmethylsulfonyl fluoride, 10 mg/ml aprotinin, 2 mg/ml leupeptin, 1 mg/ml pepstatin, at 4°C for 30 min. Lysates were clarified by centrifugation at 10,000xg at 4°C for 10 min., and protein contents were determined using the BioRad Protein determination kit. Lysates containing 20 μg protein were electrophoresed in 9% SDS-polyacrylamide gels. Proteins were transferred from gels onto nitrocellulose filters by electroblotting, and subsequently, blocked overnight at 4°C in TBST (10 mM Tris, pH 8.0, 150 mM NaCl, 0.2% Tween-20) containing 1% bovine serum albumin (fraction V). Blots were then incubated in anti-HA monoclonal antibody (12CA5) diluted 1:200 in TBST at room temperature. After four hours, the primary

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antibody was removed by washing three times for 5 min. each in TBST at temperatures identical to the primary antibody incubation. Blots were exposed to a 1:16,000 dilution of horseradish peroxidase-conjugated sheep anti-mouse immunoglobulin G. Excess secondary antibody was removed in the same manner as the primary antibody. Blots were then incubated 1-2 min. in enhanced chemiluminescence detection reagents (Amersham Inc, Arlington Heights, IL) and exposed to X-ray film (Fujifilm, Fuji Photo Film Co., LTD., Tokyo).

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Angiogenesis assay

RBE4 cell lines were harvested using trypsin-EDTA, centrifuged, and plated on collagen coated dishes at equal cell densities (50% confluence at plating) with or without bFGF (5ng/ml) RBE4 cell culture was done in the absence of 2mM Sodium Butyrate. After 3 to 5 days, cell cultures were photographed with a Nikon ELWD 0.3 phase contrast microscope on Kodak T-Max film (100 X magnification).

20 Results

Generation of RBE4 cell lines

RBE4 cell lines programmed to express the int-3, Jagged, and Notch4 proteins were generated using either the retroviral vector pLHTCX which drives expression from the CMV promoter, or by direct transfection of the cDNA in target RBE4 cells. In order to detect the int-3, and Notch4 proteins, the int-3, Jagged and Notch4 cDNA were fused at the carboxy termini to the haemagglutinin-epitope (HA), allowing us to detect int-3, Jagged and Notch4 proteins in immunoblot analysis using the anti-HA monoclonal antibody. As expected, all 3 recombinant proteins migrate with an expected molecular weight (70kd for int-3HA, 150kd for JaggedHA, and 220kd for Notch4HA). Moderate levels of recombinant protein expression is detected in the RBE4 cell lines generated. This expression level can be significantly increased by treating all RBE4

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cell line with Sodium Butyrate (2mM) which enhances transcription of the CMV promoter. Addition of Sodium Butyrate to the RBE4 angiogenic assay did not alter or enhance the observed phenotype described below, and therefor Sodium Butyrate was omitted from all RBE4 cell biological assays. As a control, RBE4 cells were generated that were programmed to express LacZ.

Morphological heterogeneity of RBE4 cell lines

RBE4 cells programmed to express LacZ or Notch4HA have an identical cell morphology when compared to wild type RBE4 cells (Fig 8). When RBE4 cells programmed to express either LacZ or Notch4 are grown in high concentrations of bFGF, they exhibit a spindle shape morphology and form modest sprouting and capillaries, a phenotype that is indistinguishable with wild type RBE4 cells grown under identical conditions (data not shown).

RBE4 cells programmed to express JaggedHA display a modest spindle shape morphology when grown in the absence of bFGF (see Fig 8). RBE4 cells programmed to express int-3HA display a striking spindle shape morphology when grown in the absence of bFGF (Fig 8). Thus, RBE4 cells programmed to express either JaggedHA or int-3 HA exhibit a morphology in the absence of bFGF that is similar (although more extensive) to the bFGF induced morphological change of wild type RBE4 cells, or RBE4 cells programmed to express either LacZ or Notch4. When RBE4 cells programmed to express Jagged or int-3 are grown in the presence of high concentrations of bFGF, the spindle shape morphology of the cells becomes even more accentuated (data not shown).

Induction of angiogenesis by activated Notch4 and Jagged

RBE4 cells programmed to express LacZ or Notch4HA growth arrest at confluency and in the absence of bFGF do not develop any three dimensional structures (Fig 8). This phenotype is identical to confluent wild type RBE4 cells

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grown in the absence of bFGF. When confluent RBE4 cells programmed to express either LacZ or Notch4 are grown in high concentrations of bFGF (5ng/ml), they form modest sprouts and capillaries, a phenotype that is indistinguishable with wild type RBE4 cells grown under identical conditions (data not shown).

RBE4 cells programmed to express JaggedHA develop sprouts and three-dimensional capillary-like structures when grown in the absence of bFGF (see Fig 8). The capillary structures formed by RBE4 cells programmed to express Jagged either float (although originating from a mount formed on the tissue culture plate) in the cell culture medium or are attached to the tissue culture plate. There is a bias in the photographs depicted in Fig 8, in favor of taking photographs of these structures that are attached to the tissue culture, plate since these are easier to focus on than on the floating capillaries that are difficult to visualize in one plain. RBE4 cells programmed to express int-3HA develop similar three-dimensional structures when grown in the absence of bFGF (Fig 8), however the extent is even greater than when compared to RBE4 cells programmed to express Jagged. Thus, RBE4 cells programmed to express either JaggedHA or int-3 HA exhibit a morphology in the absence of bFGF that is similar (although more extensive) to the bFGF induced morphological change of wild type RBE4 cells, or RBE4 cells programmed to express either LacZ or Notch4. When RBE4 cells programmed to express Jagged or int-3 are grown in the presence of high concentrations of bFGF, the development of the three dimensional capillary structures becomes even more accentuated (data not shown).

Discussion

Several conclusions of the above described experiments can be made.

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This data demonstrates that int-3 (an activated Notch4) and Jagged contain a biological activity on RBE4 cells that is

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similar to the known angiogenic agent bFGF, suggesting that this activity is an angiogenic activity.

5 This data suggests that RBE4 cells express an endogenous Notch, since RBE4 cells that are programmed to express Jagged (a mammalian Notch ligand) have a similar phenotype when compared to RBE4 cells that express a constitutive activated Notch (int-3). This is consistent with our previously published data that Notch4 is expressed in
10 endothelial cells in vivo.

This data also suggest that RBE4 cells do not express the Notch4 ligand since RBE4 cells programmed to express Notch4 do not exhibit a spindle form morphology when grown in the
15 absence of bFGF.

This data also demonstrates that over expression of Notch4 in RBE4 cells does not result in activation of the Notch4 receptor, because otherwise one would expect that these
20 cells would have a similar phenotype to those cells that express a constitutive activated form of the Notch4 receptor.

This data demonstrates that Jagged or activated Notch4
25 induced activity can synergize or cooperate with bFGF.

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References Corresponding to the Second Series of
Experiments

Roux, et al. (1994) J. Cell Physiol. 159:101-13.

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Zimrin, et al. An Antisense Oligonucleotide to the Notch
Ligand Jagged Enhances Fibroblast Growth Factor-induced
Angiogenesis in Vitro (1996) J. Biol. Chem. No. 51
271:32499-32502.

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Third Series of Experiments**Notch4 and Jagged-1 Induce Microvessel Differentiation Of Rat Brain Endothelial Cells**

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INTRODUCTION

The mouse Notch4 gene is expressed specifically in endothelial cells. Notch4/int-3, a truncated form of Notch4, acts as a constitutive activated Notch receptor. We used Rat brain microvessel endothelial cells (RBE4) as a model system to study the role of Notch4 and Jagged-1 in endothelial cell differentiation. Both Notch4/int-3 and Jagged-1 were able to induce cellular structures with morphological and biochemical properties of endothelial microvessels. Ectopic expression of full length Notch4 did not have any discernible effect in RBE4 cells. Activation of the notch signal transduction pathway was measured by the induction of endogenous Notch4 and Jagged-1 genes. The observed morphological changes to RBE4 cells correlated with endogenous Notch4 and Jagged-1 gene activation, demonstrating a link between Notch signaling and biological activity. Our observations demonstrate that Notch signaling can promote endothelial cell differentiation.

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Notch4 is a member of the Notch/lin-12 family of transmembrane receptors that are involved in cell fate determination (1,2). Like other Notch proteins, Notch4 extracellular domain is characterized by both Epidermal Growth Factor (EGF)- like repeats and lin-12/Notch repeats (LNR), and the intracellular domain contains ankyrin/cdc10 repeats (3). Analysis of invertebrate Notch/lin-12 mutants support a function for Notch/lin-12 receptors in intercellular signaling events that control cell fate. Mutants that delete the extracellular domain of C. elegans lin-12 or Drosophila Notch result in dominant gain-of-function phenotypes. The int-3 form of Notch4, Notch4/int-

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3, was identified based on its oncogenic effects in the mouse mammary gland and encodes only the transmembrane and intracellular domain of Notch4 (3-5). Notch4/int-3 behaves as a constitutively activated receptor (6-8). Similar
5 activating mutations in the Notch1 gene have been identified in T lymphoblastic leukemia and can lead to neoplastic transformation in vitro (9,10).

Drosophila Delta (11) and Serrate (12) and C. elegans Lad-2
10 (13) and Apx-1 (14) encode a family Notch/lin-12 ligands that are transmembrane proteins whose extracellular domains contain EGF-like repeats and a DSL domain (Delta-Serrate-Lag-2). Based on homology to the Drosophila ligands, Notch ligands have also been identified in vertebrates. Jagged-
15 1, a rat homologue of Drosophila Serrate, contains the hallmarks of a Notch ligand (15). Other putative mouse Notch ligands Delta-like 1 (16) and Delta-like 3 (17) have been identified and are closely related to Drosophila Delta. More recently, a human Jagged-2 gene has been
20 identified (18). Although several Notch ligands and Notch receptors have been identified in mammals, it is not clear if ligands display distinct specificities towards different receptors.

25 Several genes encoding Notch ligands or receptors are expressed in the endothelium. Notch4 is expressed primarily in endothelial cells of the vasculature of mouse embryos and adult tissues (3, 19). Notch1 and Jagged-1 are also expressed in the endothelium, as well as a variety of
30 other tissues (20-23). Recently, Jagged-1 was identified as gene induced during angiogenesis in vitro (24). A role of Notch in vascular development is suggested by analysis of mice with targeted disruption of genes encoding Notch ligands or Notch regulatory components. Mice deficient in
35 either Delta like-1 (DII1) (25,26) or Jagged-1 develop severe hemorrhages (T. Gridley and G. Weinmaster, unpublished data). Mice with targeted disruption of the

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Presenilin1 gene(26, 27), which facilitates Notch activity, display a loss of expression of Notch1, DII1 and exhibit hemorrhages in the brain and spinal cord. Evidence for involvement of Notch genes in vascular disorders has come from an analysis of a human hereditary adult onset condition causing stroke and dementia. CADASIL (cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephelopathy) is a human disorder manifested by stroke and dementia and characterized by non-amyloid and non-atherogenic angiopathy of cerebral arterioles (28). The CADASIL locus maps to the human Notch3 gene and mutations in Notch3 have been defined in CADASIL patients (29). Thus, alterations in Notch signaling may disrupt development and integrity of the vasculature.

As Notch functions in cell fate regulation in Drosophila and C. elegans, Notch receptors and their ligands expressed in endothelium may similarly control cell fate decisions during vascular development. We used the Rat brain endothelial cell line, FBE4, to study the role of Notch in endothelial cell differentiation. Ectopic expression of an activated Notch4/int-3 or of Jagged-1 in RBE4 cells induced cellular structures with morphological and biochemical properties of endothelial microvessels. Thus, activation of the Notch signaling pathway can promote endothelial differentiation.

MATERIALS AND METHODS

Immunohistochemistry

Frozen mouse kidney sections were fixed in 100% acetone and rinsed in 0.1% Triton X-100 in PBS (PBST). Tissue sections were incubated in blocking solution (5% goat serum, 3% BSA in PBS) for 1 hour. Sections were covered with 1⁰ antibody in dilution solution (1% goat serum, 3% BSA in PBS) for 12 hours at 4⁰ C. The anti-PECAM) antibody (PharMingen) was used at a concentration of 3 µg/ml, the anti-mouse Notch4

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immune-serum and pre-immune serum were diluted 500X. Tissue sections were rinsed in PBST and 2⁰ antibodies (biotinylated goat anti-rat IgG and goat anti-rabbit IgG. labeled to alkaline phosphatase, both at 1:2000 dilutions) were applied in dilution buffer for 2 hours. Biotinylated goat anti-rat IgG was detected by the peroxidase technique (Vectastain Elite ABC kit, Vector Laboratories), and alkaline phosphatase labeled goat anti-rabbit IgG was detected as previously described (30). Sections for alkaline phosphatase staining were counter stained with eosine.

cDNA's

The Notch4/int-3 cDNA corresponds to a truncated Notch4 cDNA as described previously (3). An oligonucleotide sequence encoding the haemagglutinin (HA) eptope was appended to the 3' end of the Notch4/int-3 and Notch4 cDNA's as previously described (6). The presence of each fusion was confirmed by DNA sequencing. Similarly, a Jagged-1 cDNA was modified to encode an HA-tag at the carboxy terminus.

Cell cultures and RBE4 microvessel outgrowth assay

RBE4 cells were maintained in Alpha MEM/Ham's F10 (1:1) supplemented with 2 mM glutamine, 10% fetal calf serum and penicillin/streptomycin. For microvessel outgrowth assay, RBE4 cell lines were plated at equal cell densities (50% confluence). After 3 to 5 days, cell cultures were photographed (100X or 40X magnification).

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Cell lines

HA-tagged cDNAs were inserted into the retroviral vector pLHTCX wherein hygromycin-resistance gene is controlled by the murine leukemia virus (MLV) long terminal repeat (LTR), and cDNA transcription is controlled by an internal cytomegalovirus (CMV) promoter. Distinct populations of RBE4 cells, either expressing Notch4/int-3 or Jagged-1 cDNA, were prepared by retroviral infection as described previously (6). The resultant populations, each comprised of at least 50 clones, were used in cellular and biochemical assays described below. Notch4 expressing cell lines were established by transfecting RBE4 cells directly by calcium phosphate transfection.

Immunoblot analysis

HA epitope-tagged or non-tagged Notch4/int-3, Jagged-1 and Notch4 proteins from lysates of RBE4 cells were analyzed by immunoblotting as described previously (6). Lysates containing 20 μ g protein were electrophoresed and transferred to nitrocellulose filters, and subsequently, blocked in TBST (10 mM Tris, pH 8.0, 150 mM NaCl, .02% Tween-20) containing 1% BSA (TBST-BSA). Blots were then incubated with 1⁰ antibody diluted (1:100 for 12CA5 (Berkeley Antibody Co.); 1:2000 for anti-Notch4) in TBST-BSA for 4 hours, washed in TBST, and incubated with 2⁰ antibody in TBST-BSA for 1 hour. After 3 washes, the signal was visualized by chemiluminescence (Amersham, ECL).

Histochemical enzyme assay

Gamma-glutamyl transpeptidase (GTP) activity was demonstrated histochemically in cell cultures fixed in acetone, as described previously (31): L-gamma-glutamyl-4methoxy-2-naphthylamide was used as substrate and glycylglycine as acceptor. In a simultaneous azo coupling reaction with Fastblue BB, a red azo dye is produced.

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Alkaline phosphatase (ALP) activity was determined in cell cultures fixed in citrate-buffered acetone, using the reaction mixture Sigma Kit N85, with naphtol ASMX phosphate as substrate and fast blue RR salt as diazonium salt, as previously described (31).

Northern Blot analysis

Total RNA was isolated from RBE4 cell and Northern blot analysis was performed as described previously (3). ³²P-radio labeled riboprobes were transcribed (Maxiscript, Ambion) from Notch1, Notch3, Notch4 and Jagged-1 cDNA's. Comparable amounts and integrity of the RNA were tested by hybridization with a B-actin probe.

RESULTS

Notch4 expression in Kidney endothelial cells

We previously described endothelial cell specific expression of Notch4 mRNA in mouse embryos and adult mouse lung, as analyzed *in situ* hybridization (3). To define the pattern of Notch4 protein expression, we generated an anti-Notch4 rabbit antiserum directed against a GST fusion protein containing the carboxy-terminus of Notch4 (32). The presence of endogenous Notch4 protein was analyzed in mouse kidney sections, as the kidney contains characteristic glomeruli with endothelial cell clusters in the renal cortex. We first analyzed Platelet Endothelial Cell Adhesion Molecule-1 (PECAM), an extracellular protein specifically expressed in endothelial and platelet cells. Antibodies against PECAM were used as a positive control in these experiments as they stained glomeruli (Fig. 1B). As shown in Fig. 1D, anti-Notch4 antibody specifically detects glomeruli in the kidney cortex in a similar pattern as the anti-PECAM antibody (Fig. 1B). Thus, data presented here

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demonstrates endothelial expression of Notch4 protein in the kidney.

5 The anti-Notch4 antibody was used to detect ectopically expressed Notch4 and Notch4/int-3 proteins by immunoblot analysis (Fig. 2C). Transiently transfected 293 cells expressed either HA epitope-tagged or non-epitope-tagged versions of Notch4 and Notch4/int-3. Immunoblot analysis of extracts from these cells displayed the full length
10 Notch4 and Notch4/int-3 proteins which migrate with an approximate molecular weight of 220 and 70 kD respectively (Fig. 2B and C).

RBE4 microvessel outgrowth is induced by activated Notch4 and Jagged-1
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Previous work has shown that the RBE4 cell line grown on collagen coated plates display a cobblestone morphology (31). When cultured in the presence of basic Fibroblast Growth Factor (bFGF), RBE4 cells change to a spindly
20 morphology. When grown post-confluence, bFGF induces RBE4 cells to develop multi-cellular aggregates that arise from the monolayer. These three-dimensional aggregates consist of sprouts that extend above the monolayer and organize into curvilinear and bifurcating structures, resembling
25 microvessels. bFGF-induced RBE4 microvessels contain high activity of alkaline phosphatase (ALP) and gamma glutamyl transpeptidase (GTP), which are specific enzymatic markers for differentiated endothelium and brain capillaries respectively (31).

30 RBE4 cell lines were programmed to express the HA-tagged Notch4/int-3, Jagged-1 and LacZ proteins using the retroviral vector pLHTCX. RBE4 cell lines programmed to express Notch4 were generated by direct transfection of the
35 HA-tagged Notch4 cDNA. All three recombinant proteins were detected by immunoblot analysis of the respective RBE4 cell lines and migrated with their predicted molecular weight

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(220kD for Notch4, 70kD for Notch4/int-3, 150 kD for Jagged-1, see Fig. 2D).

The consequences of activating the Notch signaling pathway
5 by ectopic expression of ligand (Jagged-1) or an activated
receptor (Notch4/int-3) was analyzed in RBE4 cells.
Control RBE4 cells expressing LacA (RBE-LacZ) growth arrest
at confluence and do not develop three dimensional
structures (Fig. 3A). When confluent RBE-LacZ cells were
10 grown with bFGF (5 ng/ml), they form modest sprouts and
microvessels (date not shown). RBE-LacZ were
indistinguishable from wild type RBE4 cells grown under
identical conditions. RBE4 cells programmed to express
either Jagged-1 (RBE-Jagged-1) or Notch4/int-3 (RBE-
15 Notch4/int-3) develop sprouts and three dimensional
capillary-like structures (Fig. 3C and D). These
structures were not dependent on the presence of bFGF.
These microvessel structures either float in the cell
culture medium (although originating from a mount formed on
20 the tissue culture plate) or are attached to the tissue
culture plate. The extent of microvessel induction by
Notch4/int-3 is far greater when compared to Jagged-1, as
demonstrated in Fig. 3E and F. RBE4 cells programmed to
express Notch4 (RBE-Notch4) do not form any microvessel
25 structures (Fig. 3B). RBE-Notch4/int-3 or RBE-Jagged-1
cells displayed a more spindle shape morphology and do not
form any microvessel structures (Fig. 3B). RBE-Notch4/int-
3 or RBE-Jagged-1 cells displayed a more spindle shape
morphology and do not form the cobblestone monolayer, as
30 observed in RBE-LacZ or RBE-Notch4 (compare Fig. 3C and D
with A or B). This morphological change is similar to when
wild type RBE4 cells are treated with bFGF (31).

Microvessel structures induced by Jagged-1 and Notch4/int-3
35 in RBE4 cells were analyzed histochemically for the
activities of two blood-brain barrier-associated enzymes,
GTP and ALP. These enzymes are specifically expressed in

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the brain vasculature, and their expression is induced by bFGF treatment of RBE4 cells (31). ALP activity was detected in microvessel structures induced by either Notch4/int-3 (Fig. 4B) or by Jagged-1 (data not shown). GTP activity was also detected in both RBE-Jagged-1 (Fig. 4C) and RBE-Notch4/int-3 (data not shown). The detection of both ALP and GTP activities was specific for microvessel structures and not found in surrounding monolayer cells. RBE4 cell lines that did not exhibit microvessels, RBE-LacZ and RBE-Notch4, did not display ALP or GTP activities (data not shown).

Activation of the Notch signal transduction pathway in RBE4 cells

Jagged-1 and Notch4/int-3 expression resulted in similar phenotypes in RBE4 cells, thus we hypothesized that RBE4 cells must express endogenous Notch receptors that can be activated by Jagged-1. Northern Blot analysis demonstrated endogenous expression of Notch1, Notch3 and Notch4 transcripts (Fig. 5A). We also found that RBE4 cells express endogenous Jagged-1 transcripts (Fig. 5A). We could not make a statement about the relative levels of expression of Notch and Jagged transcripts as each lane in Fig. 5A utilized a different riboprobe.

Activation of Notch signaling has been reported to induce endogenous genes encoding Notch receptors and ligands (18). This induction can serve as a measure of signal activation and may lead to positive feedback control of Notch genes by their ligands (33). To measure Notch signaling output in RBE4 cells, we assessed the level of endogenous Notch receptor and Notch ligand gene transcripts by northern blot analysis (Fig. 5B). RBE-Notch4/int-3 have increased levels of endogenous Notch4 (6.7 kb) and Jagged-1 transcripts (5.9 kb) (Fig. 5B, lane 2). The Notch4-specific riboprobe corresponds to the carboxy-terminal domain of Notch4, and can detect both ectopic Notch4/int-3 transcripts (1.8 kb)

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and endogenous Notch4 transcripts (6.7 kb). Notch4/int-3 expression in RBE cells specifically led to increased levels of endogenous Notch4 transcripts (Fig. 5B, lane 2) and not endogenous Notch1 (Fig. 5B, lane 2) or Notch3 transcripts (data not shown). RBE-Jagged-1 cells also have increased levels of endogenous Notch4 transcripts (Fig. 5B, lane 4). Again this activation is restricted to the endogenous Notch4 gene since the levels of Notch1 (Fig. 5B lane 4) and Notch3 (data not shown) transcripts remain unchanged. We were not able to ascertain if Jagged-1 expressing RBE4 cells up-regulated endogenous Jagged-1, since both the ectopic and endogenous Jagged-1 transcripts migrate with a similar molecular size (5.9 kb). RBE-Notch4 cells do not have increased levels of Notch1 (Fig. 5B, lane 3), Notch3 (data not shown) or Jagged-1 transcripts (Fig. 5B, lane 3). The level of Notch4 transcripts in these cells could not be evaluated since we can not distinguish by size between endogenous and ectopic Notch4 transcripts.

RBE4 cell lines were generated with retroviral vectors designed to express genes via an internal CMV promoter; however, gene expression may also be driven by the retroviral LTR leading to expression of two different sized transcripts. CMV-driven transcripts correspond to 1.8 kb for Notch4/int-3 6.7 kb for Notch4 and 5.9 kb for Jagged-1 (Fig. 5B). Larger transcripts encompassing the complete retroviral genome originate from the LTR resulting in transcripts that are 3.5 kb larger than the CMV-driven transcripts. The LTR-driven transcripts are denoted in fig. 5B (LTR) and correspond to 5.3 kb for Notch4/int-3, 10.2 kb for Notch4 and 9.4 kb for Jagged-1 (Fig. 5B). To clearly distinguish between the activated endogenous Notch4 transcripts (6.7 kb) and the LTR-driven Notch4/int-3 transcript (5.3 kb) a short exposure of a different northern blot from RBE-Notch4/int-3 cells is shown in Fig. 5B (lane 5 and 6). Fig. 5B lanes 2 and 6 both demonstrate that Notch4/int-3 activities expression of the endogenous

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Notch4 gene (6.7 kb).

DISCUSSION

5 To investigate the role of Notch4 and Jagged-1 in
endothelial cell differentiation we used an angiogenic in
vitro model system, the RBE4 cell line. Previous studies
have demonstrated that bFGF, a well characterized
angiogenic factor, induces microvessel formation and the
10 expression of markers specific for either differentiated
endothelium (ALP) or the blood-brain barrier (GTP) (31).
Ectopic expression of either Notch4/int-3 or Jagged-1 in
RBE4 cells also results in the induction of microvessel
structures that express both ALP and GTP. Full length
15 Notch4 expression did not elicit any observable phenotype.

These studies suggest that Notch4/int-3 and Jagged-1 induce
a biological activity in RBE4 cells that is similar to that
induced by a known angiogenic agent bFGF. Thus, in rat
20 brain endothelial cells, Notch activation results in an
angiogenic response characterized by microvessel formation.
Thus, Notch activation may regulate the angiogenic process
in endothelium where Notch4 is found to be expressed, such
as the vasculature of the adult kidney and lung.

25 Our data support the concept that Notch4/int-3 is a
constitutive activated Notch allele as it induces a
phenotype that is identical to when endogenous Notch
receptors are activated by Jagged-1. We also note that
30 ectopic expression of full length Notch4 in RBE4 cells does
not result in Notch signal activation, thus receptor over
expression does not lead to constitutive receptor
activation. It may also suggest that RBE4 cells do not
express a Notch4 ligand. Alternately RBE4 cells may not
35 express Notch4 ligands at levels sufficient to activate the
ectopically expressed Notch receptor. The induction of
microvessel structures by Notch4/int-3 appears to be more

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robust when compared to the induction by Jagged-1 suggesting that a constitutive activated form of the Notch4 receptor is more potent in its activity than a Notch ligand (Jagged-1).

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Since Jagged-1 is able to induce a biological activity that is similar to Notch4/int-3, we hypothesize that RBE4 cells must express endogenous Notch receptors which can be activated by Jagged-1. Northern blot analysis demonstrated that the Notch1, Notch3 and Notch4 genes are expressed in RBE4 cells. Notch1 and Notch4 are known to be expressed in endothelium *in vivo* (3, 19-23) and Notch3 may function in the vasculature (29). RBE4 cells also express Jagged-1 when analyzed by Northern blot. Thus, although RBE4 cells express endogenous transcripts that encode for both Notch proteins and one of their ligands, they do not spontaneously form microvessel structures. This observation may suggest that the levels of Notch proteins or ligands in RBE4 cells are insufficient to activate the Notch pathway.

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We demonstrated that the Notch signal transduction pathway is activated in RBE4 cells expressing either Notch4/int-3 or Jagged-1. Activation of Notch signaling results in up-regulation of the endogenous Notch4 and Jagged-1 transcripts. Thus, the observed microvessel induction of RBE4 cells correlated with Notch signal activation. Both Jagged-1 and Notch4/int-3 expression resulted in a similar up-regulation of the endogenous Notch4 gene but neither altered the levels of Notch1 and Notch3 transcripts. Activation of a particular Notch signaling pathway, in this case Notch4, led to transcriptional activation events that are specific for distinct endogenous Notch genes. The observation that Jagged-1 results in an identical transcriptional activation pattern as activated Notch4/int-3 may suggest that Jagged-1 is a Notch4 ligand.

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Previous work has demonstrated that activation of the Notch signal transduction pathway in mammalian cells inhibits differentiative or morphogenetic events. For instance, activated Notch1 and Notch2 are able to inhibit myogenic differentiation (15,34), and myeloid differentiation (35), and activated Notch4 (Notch4/int-3) is able to inhibit branching morphogenesis of mammary epithelial cells (6). In contrast, we found that activated Notch4/int-3 promoted the differentiation of RBE4 cells. Cell fate determination may involve either inhibition or promotion of differentiative steps. Our data is thus a clear demonstration that Notch signal activation can regulate cell fate decisions that involve the promotion of differentiation in mammalian cells.

Cell fate and cell differentiation decisions likely play important roles during vascular development. Although some factors have been identified that regulate vascular growth, little is known about how these or other factors may regulate cell fate decisions (36). Our data suggest that Notch proteins and their ligands can promote differentiation and may be important in regulating cell fate decisions in the developing vasculature.

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-75-

References Corresponding to the Third Series of Experiments

1. Artavanis-Tsakonas, S., Matsuno, K. & Fortini, M.E.
(1995) *Science* **268**, 225-232.
- 5 2. Baudrimont, M., Dubas, F., Joutel, A., Tournier-
Lasserre, E. & Bousser, M. G. (1993) *Stroke* **24**, 122-
125.
- 10 3. Bettenhausen, B., Hrabe de Angelis, M., Simon, D.,
Guenet, J.L. & Gossler, A. (1995) *Development* **121**,
2407-2418.
- 15 4. Bigas, A., Martin, D. & Milner, L. (1998) *Mol. Cell.*
Biol. **18**, 2324-2333.
5. Capobianco, A.J., Zagouras, P., Blaumueller, C. M.,
Artavanis-Tsakonas, S. & Bishop, J.M. (1997) *Mol Cell*
Biol **17**, 6265-6273.
- 20 6. Dunwoodie, S.L., Henrique, D., Harrison, S.M. &
Beddington, R.S. (1997) *Development* **124**, 3065-3076.
- 25 7. Ellisen, L.W., Bird, J., West, D.C., Soreng, A.L.,
Reynolds, T.C., Smith, S.D. & Sklar, J. (1991) *Cell* **66**,
649-661.
8. Fleming, R.J., Scottgale, T.N., Diederich, R.J. &
Artavanis-Tsakonas, S. (1990) *Genes Dev.* **4**, 2188-2201.
- 30 9. Foux, F., Durieu-Trautmann, O., Chaverot, N., Claire,
M., Mailly, P., Bourré, J. M., Strosberg, A. D. &
Couraud, P.O. (1994) *J. Cell. Physiol.* **159**, 101-113.
- 35 10. Franco Del Amo, F., Smith, D.E., Swiatek, P.J.,
Gendron-Maguire, M., Greenspan, R.J., McMahon, A.P. &

-76-

Gridley, T. (1992) Development 115, 737-744.

11. Gallahan, D. & Callahan, R. (1997) Oncogene 14, 1883-1890.
- 5 12. Gallahan, D., Jhappan, C., Robinson, G., Hennighausen, L., Sharp, R., Kordon, E., Callahan, R., Merlino, G. & Smith, G.H. (1996) Cancer Res. 56, 1775-1785.
- 10 13. Gallahan, D., Kozak, C. & Callahan, R. (1987) J. Virol. 61, 218-220.
14. Greenwald, I. (1994) Curr. Opin. Genet. Dev. 4, 556-562.
- 15 15. Hanahan, D. (1997) Science 277, 48-50.
16. Hrabe de Angelis, M., McIntyre II, J. & A., G. (1997) Nature 386, 717-721.
- 20 17. Hsieh, J. J., Nofziger, D. E., Weinmaster, G. & Hayward, S. D. (1997) J. Virol 71, 1938-1945.
- 25 18. Hubbard, E. J, A., Wu, G., Kitajewski, J. & Greenwald, I. (1997) Genes Dev. 11, 3182-3193.
19. Jhappan, C., Gallahan, D., Stahle, C., Chu, E., Smith, G. H., Merline, G. & Callahan, R. (1992) Genes Dev. 6, 345-355.
- 30 20. Joutel, A., Corpechot, C., Ducros, A., Vahedi, K., Chabriat, H., Mouton, P., Alamowitch, S., Domenga, V., Cecillion, M., Marechal, E., Maciazek, J., Vayssiere,

-77-

- C., Cruaud, C., Cabanis, E. A., Ruchoux, M. M.,
Weissenbach, J., Bach, J. F., Bousser, M. G. &
Tournier-Lasserre, E. (1996) Nature 383, 707-710.
- 5 21. Lindsell, C.E., Boulter, J., diSibio, G., Gossler, A.
 & Weinmaster, G. (1996) Molecular & Cellular
 Neurosciences 8, 14-27.
22. Lindsell, C.E., Shawber, C.J., Boulter, J. &
10 Weinmaster, G. (1995) Cell 80, 909-917.
23. Luo, B., Aster, J.C., Hasserjian, R.P., Kuo, F. &
 Sklar, J. (1997) Mol Cell Biol 17, 6057-6067.
- 15 24. Mello, C.C., Draper, B.W. & Priess, J.R. (1994) Cell
 77, 95-106.
25. Myat, A., Henrique, D., Ish-Horowicz, D. & Lewis, J.
 (1996) Dev. Biol. 174, 233-247.
- 20 26. Reaume, A. G., Conlon, R.A., Zirngibl, R., Yamaguchi,
 T.P. & Rossant, J. (1992) J. Dev. Biol. 154, 377-387.
27. Shen, J., Bronson, R. T., Chen, D. F., Xia, W.,
25 Selkoe, D. J. & Tonegawa, S. (1997) Cell 89, 629-639.
28. Sirayoshi, Y., Yuasa, Y., Suzuki, T., Sugaya, K.,
 Kawase, E., Ikemura, T. & Nakatsuki, N. (1997) Genes
 Cells 2, 213-224.
- 30 29. Tax, F.E., Yeager, J.J. & Thomas, J.H. (1994) Nature
 368, 150-154.
- 35 30. Uyttendaele, H., Marazzi, G., Wu, G., Yan, Q.,
 Sassoon, D. & Kitajewski, J. (1996) Development 122,
 2251-2259.

-78-

31. Uyttendaele, H., Soriano, J.V., Montesano, F. &
Kitajewski, J. (1998) Dev. Biol. 196, in press.
32. Vassin, H., Bremer, K.A, Knust, E. & Campos-Ortega,
5 J.A. (1987) EMBO J. 6, 3431-3440.
33. Wilkinson, H. A., Fitzgerald, K. & Greenwald, I.
(1994) Cell 79, 1187-1198.
- 10 34. Wong, P.C., Zheng, H., Chen, H., Becher, M. W.,
Siringathsinghji, D. J. S., Trumbauer, M. E., H.Y.,
C., Price, D. L., Van der Ploeg, L. H. T. & Sisodia,
S. S. (1997) Nature 387, 288-292.
- 15 35. Zhang, Q, Ahuja, H.S., Zakeri, Z. F. & Wolgemuth, D.J.
(1997) Dev. Biol 183, 222-233.
36. Zimrin, A. B., Pepper, M. S., McMahon, G. A., Nguyen,
F., Montesano, R. & Maciag, T. (1996) J. Biol. Chem.
20 271, 32499-32502.

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What is claimed is:

1. An isolated nucleic acid encoding a Notch 4 protein.
- 5 2. An isolated nucleic acid of claim 1, wherein the nucleic acid is DNA, cDNA, synthetic DNA or RNA.
3. An isolated nucleic acid of claim 1, wherein the nucleic acid is genomic DNA.
- 10 4. An isolated nucleic acid of claim 2, having a sequence substantially the same as the sequence set forth in figure 9.
- 15 5. An isolated nucleic acid of claim 1, encoding an amino acid sequence substantially the same as the sequence set forth in figure 1.
- 20 6. An isolated nucleic acid encoding an activated Notch4 protein.
7. An isolated nucleic acid of claim 6, wherein the nucleic acid is DNA, cDNA, synthetic DNA or RNA.
- 25 8. An isolated nucleic acid of claim 6, wherein the nucleic acid is genomic DNA.
9. A nucleic acid of at least 12 nucleotides capable of specifically hybridizing with a sequence uniquely
30 present within the sequence of the nucleic acid of claim 1 or 6.
10. The nucleic acid of claim 9, wherein the nucleic acid is DNA or RNA.
- 35 11. A vector which comprises the isolated nucleic acid of claim 1 or 6.

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12. The vector of claim 11, wherein the isolated nucleic acid is operatively linked to a regulatory element.
13. A plasmid vector of claim 11.
- 5 14. The plasmid of claim 12, designated pBS-Notch4 (ATCC Accession No.).
- 10 15. A host vector system for the production of a polypeptide comprising the vector of claim 12 in a suitable host.
- 15 16. The host vector system of claim 15, wherein the suitable host is a prokaryotic or eukaryotic cell.
17. A method of transforming host cells comprising transfecting the host cells with the vector of claim 12.
- 20 18. Transformed host cells produced by the method of claim 17.
- 25 19. Transformed host cells of claim 18, wherein the host cells when not transformed do not express Notch4 protein.
20. Transformed host cells of claim 18, which express Notch4 protein.
- 30 21. The host vector system of claim 15, wherein the suitable host is a bacterial, yeast, plant, or animal cell.
- 35 22. A method of producing a polypeptide comprising using the host vector system of claim 15 under conditions permitting production of the polypeptide and recovering the polypeptide so produced.

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23. A purified, Notch4 protein.
24. A purified, activated Notch4 protein.
- 5 25. A polypeptide encoded by the isolated nucleic acid of claim 1 or 6.
- 10 26. An antisense oligonucleotide having a sequence capable of specifically hybridizing to an mRNA encoding the Notch4 protein of claim 1 so as to prevent translation of the mRNA molecule.
- 15 27. An antisense oligonucleotide having a sequence capable of specifically hybridizing to an mRNA encoding an activated Notch4 protein of claim 6 so as to prevent translation of the mRNA molecule.
- 20 28. An antisense oligonucleotide having a sequence capable of specifically hybridizing to the genomic DNA molecule of claim 3 or 8.
- 25 29. An antibody capable of specifically binding to Notch 4 protein, but not to activated Notch4 protein.
- 30 30. An antibody capable of specifically binding to activated Notch4 protein but not to Notch4 protein.
- 35 31. An antibody capable of competitively inhibiting the binding of the antibody of claim 29 to Notch4 protein.
32. An antibody capable of competitively inhibiting the binding of the antibody of claim 30 to activated Notch4 protein.
33. A monoclonal antibody of claim 29, 30, 31 or 32.

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34. A pharmaceutical composition comprising an amount of the oligonucleotide of claim 26 effective to reduce expression of Notch4 protein within a cell and a pharmaceutically acceptable carrier, wherein the composition is capable of passing through a cell membrane.
35. A pharmaceutical composition comprising an amount of the oligonucleotide of claim 27 effective to reduce expression of an activated Notch4 protein within a cell and a pharmaceutically acceptable carrier, wherein the composition is capable of passing through a cell membrane.
36. A pharmaceutical composition of claim 34 or 35, wherein the oligonucleotide is coupled to a substance which inhibits mRNA translation.
37. A pharmaceutical composition of claim 34 or 35, wherein the pharmaceutically acceptable carrier comprises a structure which binds to a receptor on a cell capable of being taken up by the cell after binding to the structure.
38. The pharmaceutical composition of claim 37, wherein the structure of the pharmaceutically acceptable carrier is capable of binding to a receptor which is specific for a selected cell type.
39. A pharmaceutical composition which comprises an amount of the antibody of claim 29 effective to block binding of a ligand to Notch4 protein and a pharmaceutically acceptable carrier.
40. A method for determining whether a ligand can specifically bind to Notch4 protein comprising the steps of:

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- a) contacting Notch4 protein with the ligand under conditions permitting formation of specific complexes between Notch4 protein and known Notch4 protein-binding ligands;
 - 5 b) determining whether complexes result from step (a), the presence of such complexes indicating that the ligand specifically binds to Notch4 protein.
- 10 41. The method of claim 40, wherein the Notch4 protein in step (a) is reconstituted in liposomes.
42. The method of claim 40, wherein the ligand is a chemical not previously known to be a ligand which can
15 specifically bind to Notch4 protein.
43. A ligand determined by the method of claim 42.
- 20 44. A method for determining whether a ligand is a Notch4 protein agonist which comprises contacting a cell transfected with and expressing Notch4 protein with the ligand under conditions permitting activation of a functional Notch4 protein response by ligands known to be agonists of Notch4 protein, and detecting
25 whether a functional increase in Notch4 protein activity occurs so as to determine whether the ligand is a Notch4 protein agonist.
- 30 45. A method for determining whether a ligand is a Notch4 protein antagonist which comprises contacting a cell transfected with and expressing Notch4 protein with the ligand under conditions permitting activation of a functional Notch4 protein response by ligands known to be agonists of Notch4 protein, and detecting
35 whether a functional decrease in Notch4 protein activity occurs so as to determine whether the ligand is a Notch4 antagonist.

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46. A pharmaceutical composition which comprises an amount of a Notch4 protein agonist effective to increase the activity of a Notch4 protein within a cell and a pharmaceutically acceptable carrier.
- 5
47. The pharmaceutical composition of claim 46, wherein the agonist comprises a peptide fragment of Jagged protein capable of increasing the activity of Notch4 protein.
- 10
48. A pharmaceutical composition which comprises an amount of a Notch4 protein antagonist effective to decrease activity of Notch4 protein within a cell and a pharmaceutically acceptable carrier.
- 15
49. A method of modulating angiogenesis in a subject comprising administering to the subject an amount of agonist or antagonist of Notch4 protein effective to promote or inhibit angiogenesis in the subject.
- 20
50. A method of promoting angiogenesis in a subject comprising administering to the subject an amount of Notch4 protein agonist effective to promote angiogenesis in the subject.
- 25
51. A method of inhibiting angiogenesis in a subject comprising administering to the subject an amount of Notch4 protein antagonist effective to inhibit angiogenesis in the subject.
- 30
52. A method of promoting angiogenesis comprising transducing selected cells, wherein the cells express activated Notch4 protein in an amount sufficient promote angiogenesis in the cells.
- 35
53. A method of promoting angiogenesis comprising transducing selected cells which express Notch4

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protein, wherein the cells express a Notch4 ligand in an amount sufficient to promote angiogenesis in the cells.

- 5 54. A method of promoting angiogenesis comprising transducing selected cells, wherein the cells express a Notch4 protein and a Notch4 ligand in an amount sufficient to promote angiogenesis in the cells.
- 10 55. The method of claim 53 or 54, wherein the Notch4 protein ligand is Jagged protein or a peptide fragment thereof capable of increasing the activity of Notch4 protein.
- 15 56. A method of inhibiting angiogenesis comprising administering to cells expressing Notch4 protein an amount of an antibody of claim 29 effective to block binding of a ligand to Notch4 protein.
- 20 57. A method of inhibiting angiogenesis comprising administering to cells expressing Notch4 protein an amount of a fragment of antibody of claim 29 effective to block binding of a ligand to Notch4 protein.
- 25 58. A method of treating an abnormality in a subject wherein the abnormality is alleviated by inhibiting angiogenesis comprising blocking Notch4 protein signaling in the subject.
- 30 59. The method of claim 58 comprising administering to the subject an amount of an antibody of claim 29 effective to block binding of a ligand to Notch4 protein.
- 35 60. The method of claim 58 comprising administering to the subject an amount of a fragment of an antibody of claim 29 effective to block binding of a ligand to Notch4 protein.

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61. The method of claims 58, 59 or 60 wherein the abnormality is a solid tumor, hemangioma, hemangiosarcoma or Kaposi's Sarcoma.
- 5 62. A method of treating an abnormality in a subject wherein the abnormality is alleviated by promoting angiogenesis comprising stimulating the Notch4 signaling pathway.
- 10 63. The method of claim 62 comprising transducing selected cells within the subject, wherein the cells express activated Notch4 protein.
- 15 64. The method of claim 62 comprising transducing selected cells within the subject expressing a Notch4 protein such that the cells express a Notch4 protein ligand.
- 20 65. The method of claim 64 wherein the ligand is Jagged protein or a peptide fragment thereof capable of increasing the activity of Notch4 protein.
- 25 66. The method of claim 62 comprising transducing selected cells within the subject such that the cells express a Notch4 protein and a Notch4 protein ligand.
- 30 67. The method of claim 66 wherein the ligand is Jagged protein or a peptide fragment thereof capable of increasing the activity of Notch4 protein.
- 35 68. The method of claims 62, 63, 64, 65, 66 or 67 wherein the abnormality is an ischemic disorder, gangrene, diabetes ulceratis, chronic ulceration, Cerebral Autosomal Dominant Arteriopathy with Subcortical Infarcts and Leukoencephalopathy (CADASIL), Vascular Dementia or a wound.

FIG. 1A

| | | | | |
|-----|----|---|----|--|
| 1 | 1 | MQPQLLLLLPLNFPVILTR | 2 | |
| 81 | 3 | CGGSPPCANGGTCLRLSRGQICQCAPGLGETCQFPDPCRDTOICKNGGSCQAL | 4 | |
| 161 | 5 | LPTPPSSRSPTSLTPHFSCTCPSGFTGDR | 6 | |
| 241 | 7 | QTHILEELCPPSFCNSGGHCYVQASGRPQCSCEPGWTCGEQCQLRDFCSAN | 8 | |
| 321 | 9 | PCANGGVLATYPQIQCRCPPGFEGHTCERDINECFLEPGCPQGTSCNHTLGSYQCLCPVGQEGPQCKLRKGACPFQSGC | 10 | |
| 401 | 11 | LNGGTCQLVPEGHSTFHLCLCPPGFTGLDCENMPDDCVRHQCQNGATCCLDGLDITYTCPKTKWGWDCSEDI DECEARCP | 12 | |
| 481 | 13 | PRCRNGGTCQNTAGSTFICVVCVSGWGGAGCEENLDDCAAAATCAPGSTCIDRVGSFSCLCPPGRTGLLCHIEDMCLSQPCHV | 14 | |
| 561 | 15 | NAQCSTNPLTGSTLICIQPGYSGSTCHQDLDECQMAQQGSPSPCEHGGSCINTPGSFNCLCLPGYTGSRCEADHNECLSQP | 16 | |
| 641 | 17 | CHPGSTCLDLLLATFHICLCPPGLEGRRLCEVEVNECTSNPCLNQAACHDLLNGFQCLCLPGFTGARCEKDHDECSSTPCANG | 18 | |
| 721 | 19 | GRCRDQPGAFYCECLPGFEGPHCEKEVDECLSDPCPVGASCLDLPGAFFCLCRPGFTGQLCEVPLCTPNMCQPGQQCQGG | 20 | |
| 801 | 21 | EHRAPCLCPDGSPPGCVPAEDNCPCHHGHICQRLCVCDEGWTGPECETELGGCISTPCAHGGTCHPQPSGYNCTCPAGYMG | 22 | |
| 881 | 23 | LTCSEEVTAHSGPCLNGGSCSIRPEGYSCTCLPSHTGRHCQTAVDHCVSA | 24 | |
| 961 | 25 | SCLNGGTCVNKPGTFFCLCATGTFQGLHCE | 26 | |
| | 27 | EKTNPSCADSPCRNKATCQDTPRGARCLSPGYTGSSCQTLIDLCAKPCPHTARCLQSGPSFQCLCLQGWGTGALCDFPL | | |
| | 29 | SCQKAAMSQGI EISGLCQNGGLCIDTGSSYFCRCPPGFQGLCQDNVNPCEPNPCHHGSTCVPPQPSGYVCQCAPGYEGQN | | |
| | 31 | CSKVLDAQSQSPCHNHGTCTSRPGGFHCACPPGFVGLRCEGDVDECLDRPCHPSGTAACHSLANAFYCQCLPGHTGQRCE | | |

EGF-like
repeats

FIG. 1B

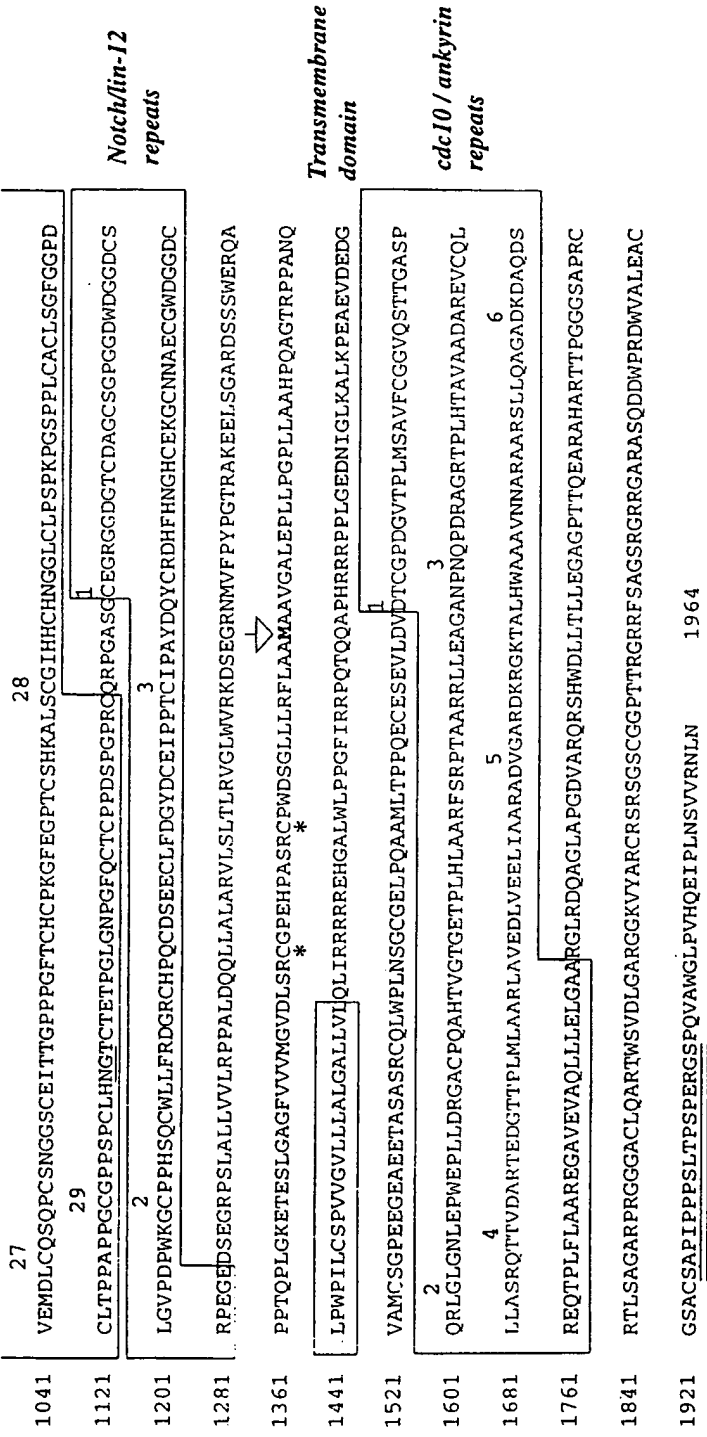
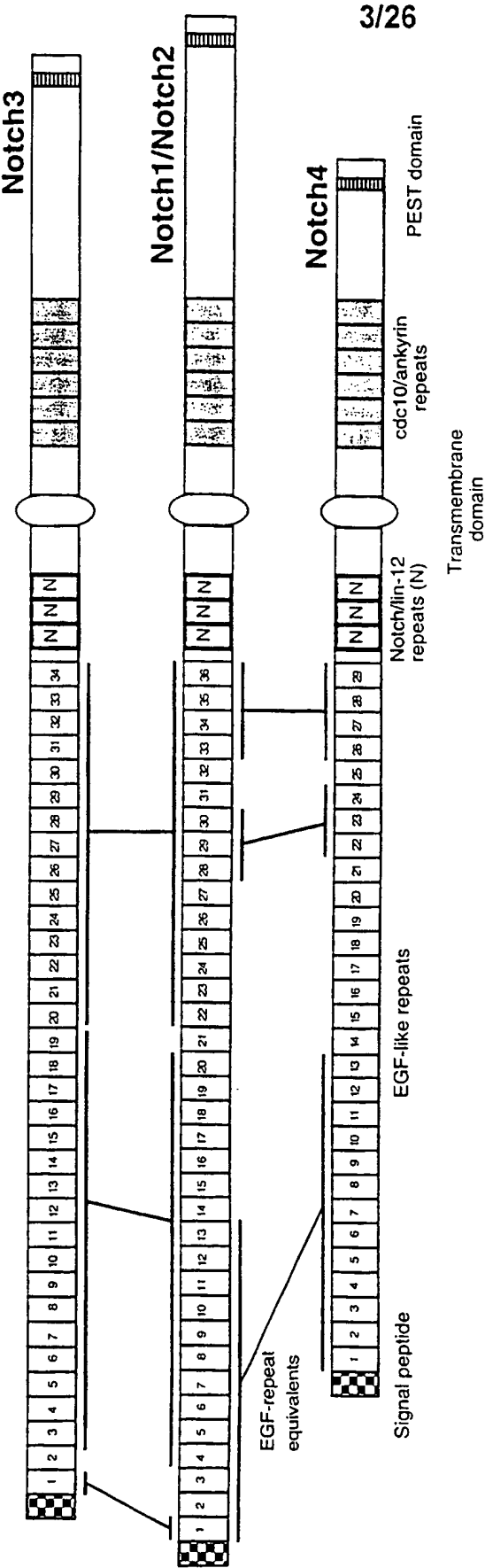
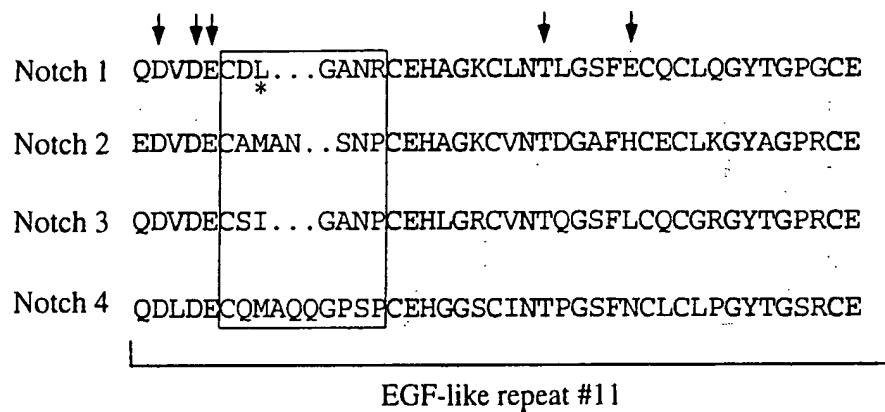


FIG. 2



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FIG. 3



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FIG. 4A

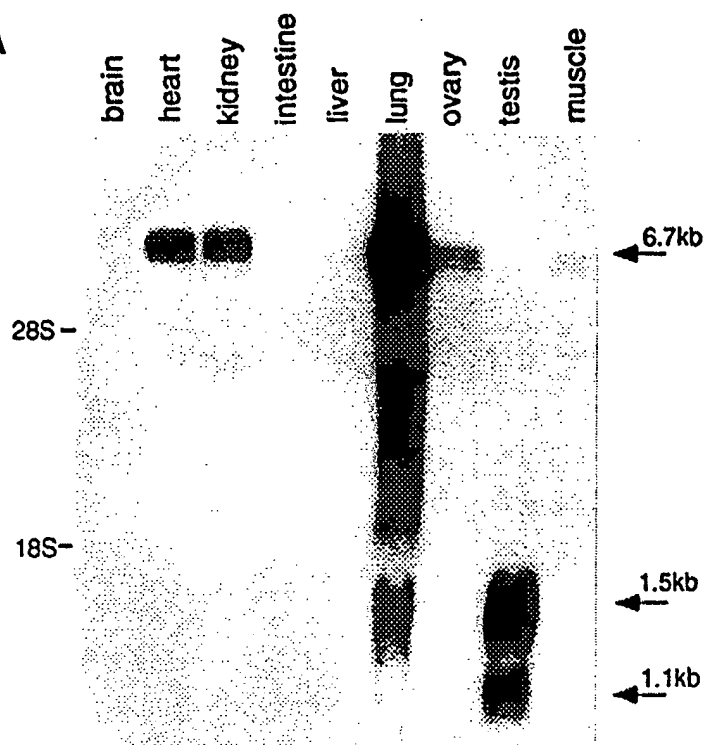


FIG. 4B



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FIG. 5A

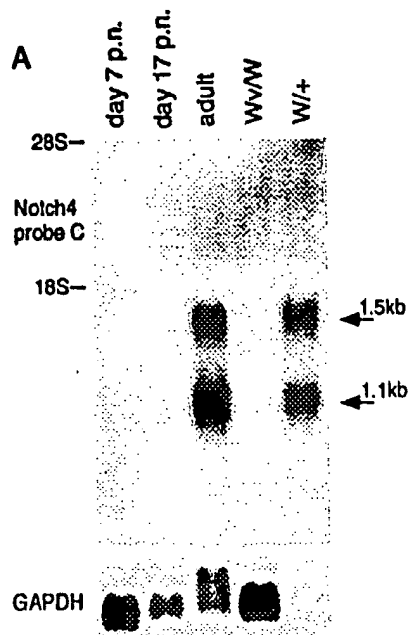


FIG. 5B

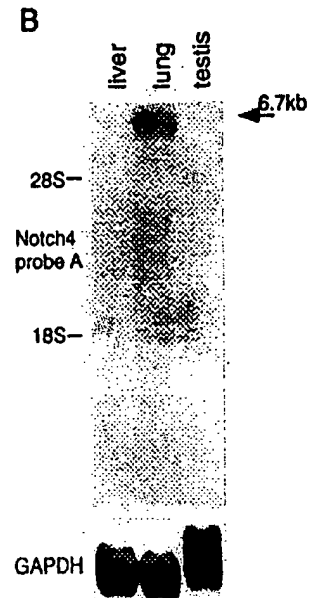
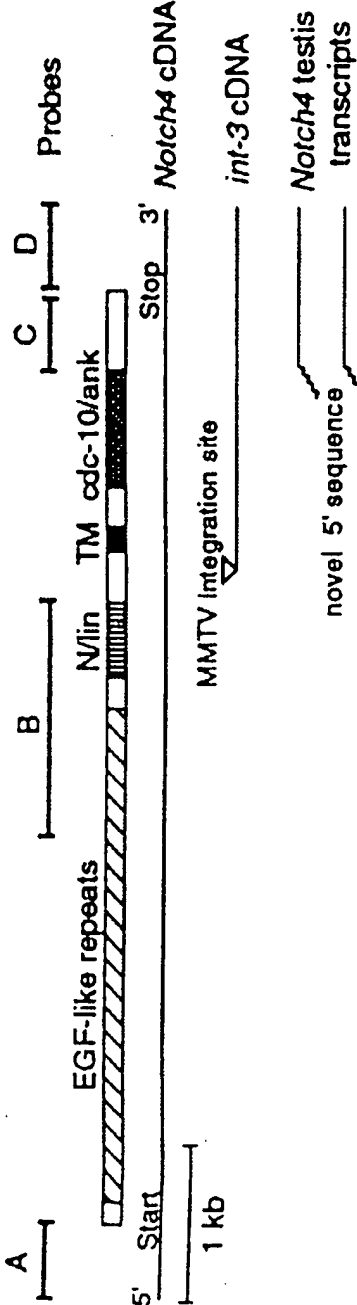


FIG. 5C



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FIG. 6B



FIG. 6D

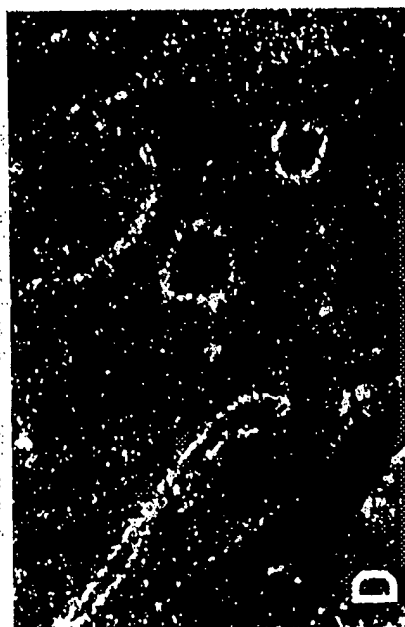


FIG. 6A

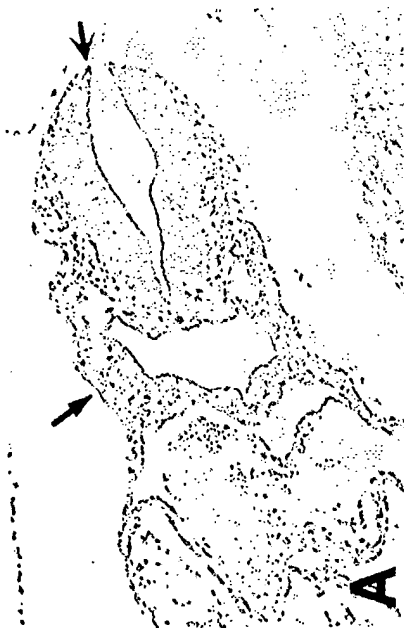
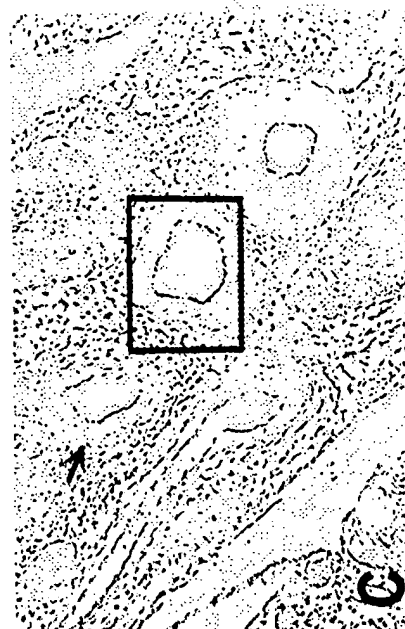


FIG. 6C



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FIG. 6F

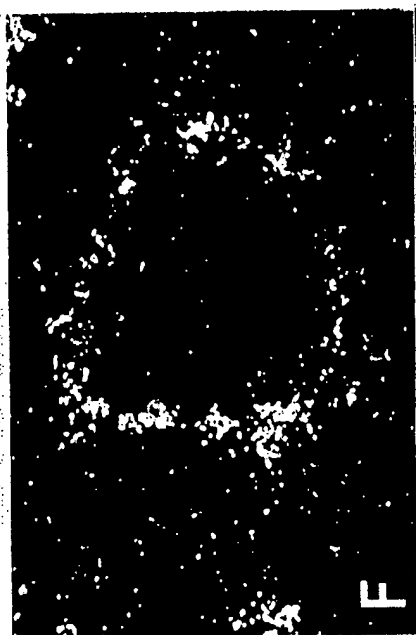
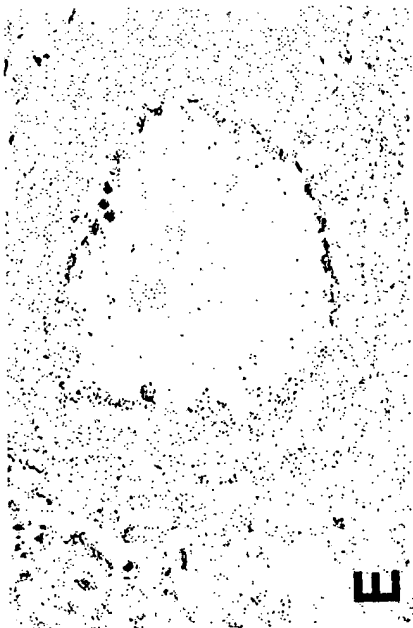


FIG. 6E



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FIG. 7B

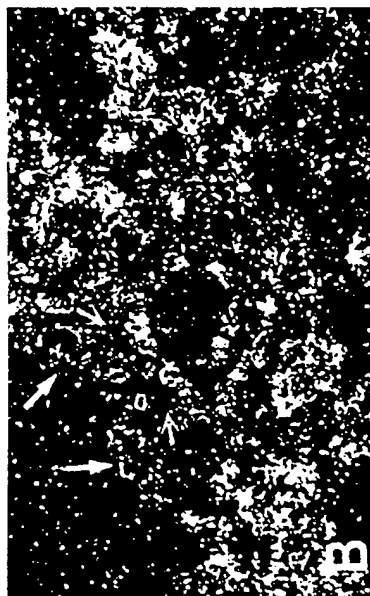
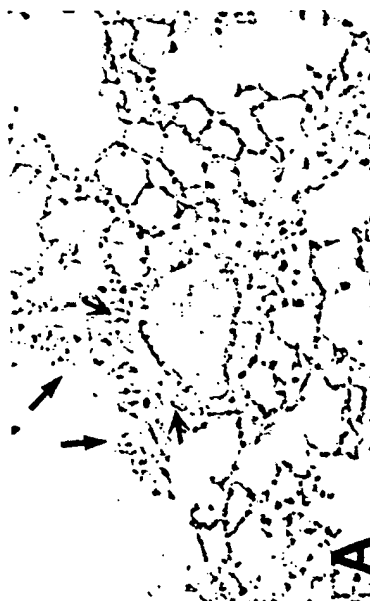


FIG. 7A



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FIG. 8A

FIG. 8B

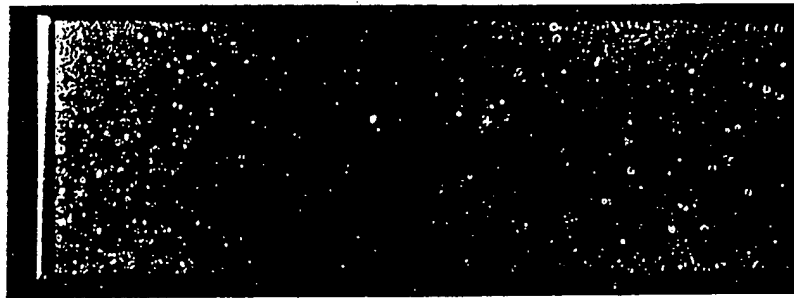
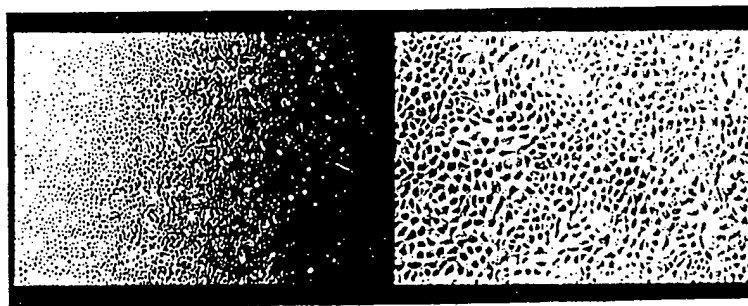


FIG. 8C

FIG. 8D



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FIG. 8E



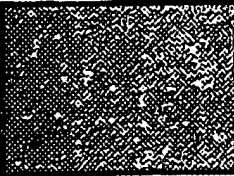
FIG. 8F



FIG. 8G



FIG. 8H



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FIG. 8I

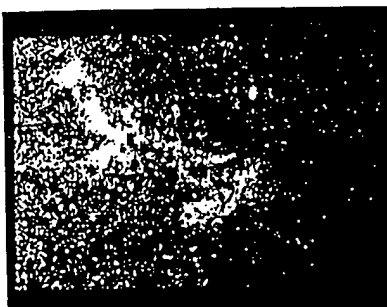


FIG. 8J

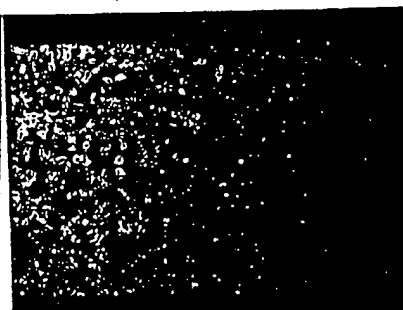


FIG. 8K

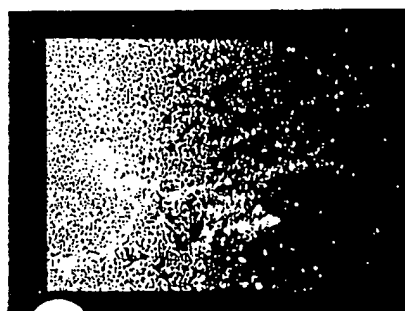


FIG. 8L



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FIG. 8M

FIG. 8N

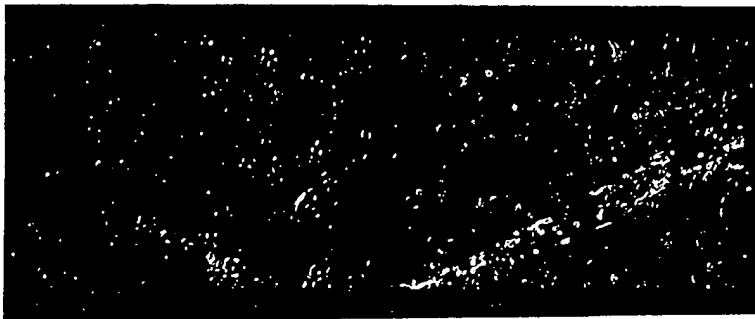
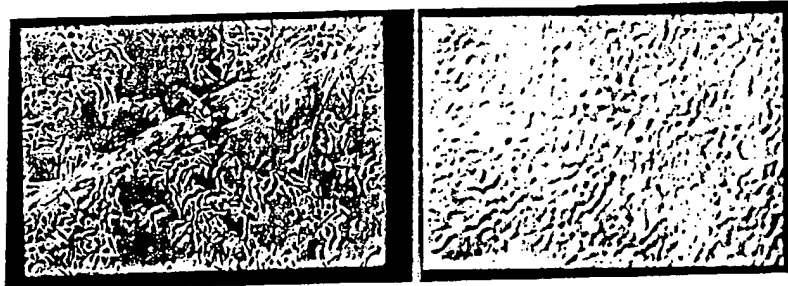


FIG. 8O

FIG. 8P



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FIG. 8Q

FIG. 8R

FIG. 8S

FIG. 8T



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FIG. 8U

FIG. 8V



FIG. 8W

FIG. 8X



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FIG. 9A

1 GGGAAACAG GTGTGTTTCT GCCTTTTACT TCTAACTTGG AATACCTTAC CCAATCCCAG
61 TTCTTCATCT CCTCTGAGAA GTTATAGGGT TAAAAATATT GTCTTCTTAC ATCAGCAGAT
121 ATATGACAA GGAAGAGATC CTTTGGTCAG CTCTAGTAAT CTGGCTTTGT CCCCCTTAGG
181 GAATAGACTT TGGCCTGAGG GAACAGCTCA GACTGAGCG TGCAGCAGGC TCAGGAGGAA
241 GAAGGCGGT AGAAGCAGAG GAAGTGGCCT TGCCTGGCCA CAAGGCTCT GAGGGTCCC
301 GCCTGAAGAG GGAGAGGAGA TCCGGGCCAG GGGCAGGTGC TCTGGAATGC AGCCCCAGTT
361 GCTGCTGCTG CTGCTCTTGC CACTCAATT CACTGTATC CCCTGTATC CTGACCCAGAG AGCTTCTGTG
421 TGGAGGATCC CCAGAGCCCT GTGCCAACGG AGCACCTGC CTGAGGCTAT CTCGGGGACA
481 AGGGATCTGC CAGTGTGCC CAGTGTGCC CTGGATTCT GGGTGAGACT TGCCAGTTTC CTGACCCCTG
541 CAGGGATACC CAACTCTGCA AGAATGGTGG CAGCTGCCAA GCCCTGCTCC CCACACCCCC
601 AAGTCCCCGT AGTCCCTACTT CTCCACTGAC CCTCACTTC TCCGTGCACCT GCCCCCTGCG
661 CTTACCCGGT GATCGATGCC AAACCCATCT GGAAGAGCTC TGTCCACCTT CTTTCTGTTC
721 CAACGGGGT CACTGCTATG TTCAGGCCCTC AGCCGCCCA CAGTGTCTCT GCGAGCCTGG
781 GTGGACAGGT GAGCAATGCC AGCTCCGAGA CTTCTGCTCA GCCAACCCCT GTGCCAAACGG
841 AGGCGTGTGC CTGGCCACAT ACCCCACAGT CCAGTGCCGC TGTCCACCTG GGTTCGAGGG
901 TCACACCTGT GAACGCGACA TCAACGAGTG CTTCCTGGAG CCGGAGCCCT GCCCTCAGGG
961 CACCTCCCTGC CATAACACCT TGGGTTCCCTA CCAGTGTCTC TGCCCTGTGG GGCAGGAAGG
1021 TCCCCAGTGC AAGCTCAGGA AGGGAGCCTG CCTCCTGGA AGTGTCTCA ATGGGGGCAC
1081 CTGCCAGCTG GTCCCAGAGG GACACTCCAC CTTTCATCTC TGCCCTCTGT CCCCAGGTTT
1141 CACGGGGCTG GACTGTGAGA TGAACCCAGA TGACTGTGTC AGGCACCAT GTGAGAACGG
1201 GGCCACCTGT CTGGATGGGC TGGATACCTA CACCTGCCCC TGCCCCAAGA CATGGAAGGG
1261 CTGGGACTGC TCTGAAGATA TAGATGAATG TGAAGCCCCG GTCCCCCTC GCTGCAGGAA
1321 CGGTGGCACC TGCCAGAACA CAGCTGGCAG CTTTCACTGT GTGTGCGTGA GTGGCTGGGG
1381 AGGTGCAGGC TGTGAGGAGA ACCTGGATGA CTGTGCAGCT GCCACCTGTG CCCCAGGATC
1441 CACCTGCATC GACCGTGTGG GCTCTTTCTC CTGCCCTCTGC CCACCTGGAC GCACAGGCCT
1501 CCTGTGCCAC CTGGAAGACA TGTGTTTGTG TCAGCCGTGC CACGTGAATG CCCAGTGCAG
1561 CACCAACCCT CTGACAGGCT CCACCCTCTG CATATGCCAG CCTGGCTACT CAGGATCCAC
1621 CTGTACCAA GATCTGGATG AGTGCCAAAT GGCCCAAGCA GGACCCAGTC CCTGCGAACA
1681 TGGGGGGTCC TGCATCAACA CCCCCTGGCTC CTTCAACTGC CTCTGCCCTG CTGGTTACAC

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FIG. 9B

1741 GGGCTCCCGC TGTGAAGCTG ACCACAATGA GTGCCTGTCA CAGCCCTGCC ACCCAGGCAG
 1801 CACCTGCCCTG GACCTGCTTG CAACCTTCCA CTGCCCTCTGC CCACCAGGCT TGGAAAGGGAG
 1861 ACTCTGTGAG GTGGAGGTCA ATGAGTGCAC CTCATAATCCC TGCCCTGAACC AAGCTGCCTG
 1921 CCATGACCTG CTC AACGGCT TCCAGTGCCT CTGCCCTTCTT GGATTCACCG GCGCCCGATG
 1981 TGAGAAAGAC ATGGACGAGT GTAGCAGCAC CCCCTGTGCC AATGGGGGGC GCTGCCGAGA
 2041 CCAGCCCTGGA GCCTTCTACT GCGAGTGTCT GTGACCCCTG TCCCGTGGGA GCCAGCTGTC TTGATCTCCC
 2101 AGAAGTGGAC GAATGTCTGA GTGACCCCTG GCCGTCCCTG TTTCACAGGT CAACTTTGTG AGGTTCCCTT
 2161 CGGAGCATTC TTCTGTCTCT GCGTCCCTGG AACATGCCAA GGTCAAGAAC ACAGAGCCCC
 2221 GTGCACCCCC AACATGTGCC GTCCCTGGCTG TGTTCCTGCC GAGGACAACT GCCCCTGTCA
 2281 CTGCCCTCTGC CCTGACGGAA CCTGTGTGTG GCATCTCCAC ACCCTGTGCC CATGGGGGA CCTGCCACCC
 2341 CCATGGCCAT TGCCAGAGAT CTGGGTGGCT GTACCTGCC TGCAGGCTAC ATGGGGTTGA CCTGTAGTGA
 2401 CGAGACAGAA CTGGGTGGCT GCTACAACT GTACCTGCC CAGGGCCCTG TCTCAATGGT GGCTCCTGCA GCATCCGTC
 2461 ACAGCCATCT GGCTACAACT GCTGTCACT CAGGGCCCTG GCCTTCCAAG TCACACAGGT CGCCACTGCC AGACTGCCGT
 2521 GGAGGTGACA GCTGTCACT CAGGGCCCTG CGTGCACCT CGTGTGCCA CTGGGGTACC TGTGTGAACA AGCCTGGCAC
 2581 TGAGGGCTAT TGAGGGCTAT TGAGGGCTAT TGAGGGCTAT TGAGGGCTAT TGAGGGCTAT TGAGGGCTAT TGAGGGCTAT
 2641 GGAGGGCTAT TGAGGGCTAT TGAGGGCTAT TGAGGGCTAT TGAGGGCTAT TGAGGGCTAT TGAGGGCTAT TGAGGGCTAT
 2701 TTTCTTCTGC CTCTGTGCCA CTCTGTGCCA CTGGCTTCCA GGGCTGTCAC TGTGAGGAGA AGACTAACCC
 2761 CAGCTGTGCA GACAGCCCTT GCATATACAGG AAGCAGCTGC CAGACTCTGA TAGACTTTGTG
 2821 CCGCTGCCCTC TGACAGCCCTG CCCTGTCCAC ACACCTGCTG ATGCCCTCCAG AGTGGGCCCT CGTTCCAGTG
 2881 TGCCCGGAAG CCCTGTCCAC CAGGGATGGA CAGGGCTCT CTGTGACTTC CCACCTGTCTT GCCAGAAGGC
 2941 CCTGTGCCCTC CAGGGATGGA AGATCTCTGG CCTGTGCCAG AATGGAGGCC TCTGTATTGA
 3001 CGCGATGAGC CAAGGCATAG GCGCTGCCC TCCCTGGATT CCAAGCAAGT TATGCCAGGA
 3061 CACGGGCTCC TCCTATTCTT CCAATCCCTG CCATCAGGG TCTACCTGTG TGCCCTCAGCC
 3121 TAATGTGAAC CCCTGCGAGC GTGCCCCCAGG CTATGAGGGA CAGAAGTACT CAAAAGTACT
 3181 CAGTGGCTAT GTCTGCCAGT CAGTCCCAGC CCTGCCACAA CCACGGAACC TGTACCTCCA GGCCCTGGAGG
 3241 TGACGCTTGT CAGTCCCAGC CAGGCTTCTG GGGACTGCG CACTGCAGCT TGCCACTCTT TAGCCAACGC
 3301 CTTCCACTGT GCCTGCCCTC CAGGCTTCTG GGGACTGCG CACTGCAGCT TGCCACTCTT TAGCCAACGC
 3361 GTGTCTGGAC CGGCCCTGTC ACCCTCGGG CACTGCAGCT TGCCACTCTT TAGCCAACGC

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FIG. 9C

3421 CTTCTACTGC CAGTGTCTGC CTGGGCACAC AGGCCAGCGG TGTGAGGTGG AGATGGACCT
 3481 CTGTCAGAGC CAACCCTGCT CCAATGGAGG ATCCTGTGAG ATCACAACAG GGCCACCCCC
 3541 TGGCTTCACC TGTCACTGCC CCAAGGGTTT TGAAGGCCCC ACCTGCAGCC ACAAAGCCCT
 3601 TTCTCTGCGC ATCCATCACT GCCACAATGG AGGCCTATGT CTGCCCCCCC CTAAGCCAGG
 3661 GTCACCACCG CTCTGTGCCT GCCTCAGTGG TTTTGGGGGC CCTGACTGTC TGACACCTCC
 3721 AGCTCCACCG GGCTGCGGTC CCCCCTCACC CTGCCTGCAC AATGGTACCT GCACTGAGAC
 3781 CCCTGGGTTG GGCAACCCCG GCTTTCAATG CACCTGCCCT CCTGACTCTC CAGGGCCCCG
 3841 GTGTCAAAGG CCAGGGGCAA GTGGGTGTA GGGCCGAGGT GGTGATGGA CCTGCGATGC
 3901 TGGCTGCAGT GGCCAGGAG GAGACTGGA TGGAGGGGAC TGTTCCTGG GGTCCCCAGA
 3961 CCCCTGGAAG GGCTGTCCCC CGCATTCCTT CTGTTCCGGG ACGGACGGTG
 4021 TCACCCGCGAG TGTGACTCTG AGGAGTGTCT CTTTGATGGC TACGACTGTG AAATCCCTCC
 4081 AACCTGCATC CCAGCCTATG ACCAGTACTG CCGAGATCAC TTCCACAACG GGCACCTGTGA
 4141 GAAAGGCTGC AATAACGCTG AATGTGGCTG GGACGGGGGA GACTGCAGAC CAGAAGGGGA
 4201 AGACTCAGAG GGGAGGCCCT CCCTGGCCCT GCTGTGGTG CTGAGGCCCC CAGCCCCTGA
 4261 TCAGCAGCTG CTTGCCCTGG CACGAGTGCT GTCCCTGACT CTGAGGGTCG GTCTCTGGGT
 4321 GAGGAAGGAC AGTGAAGGA GGAACATGGT GTTCCCCCTAT CCTGGGACCC GGGCCAAAGA
 4381 GGAGCTGAGT GGAGCTAGGG ATTCCCTCTC ATGGGAAAGA CAAGCCCCCTC CCACTCAGCC
 4441 CCTGGGCAAG GAGACAGAGT CTCTTGGTGC AGGTTTGTG GTAGTGATGG GAGTGGATCT
 4501 GTCCCGCTGT GTCCCGGAAC ATCCTGCGTC CCGCTGCCCC AGCTCTGGAG CCCCTGCTGC
 4561 GCGCTTCCTT GCAGCAATGG CAGCAGTGGG AGCTCTGGAG GCGCTGCTGC CTGGACCCCT
 4621 GCTGGCGGCT CACCCTCAAG CAGGACCCAG GCCCCCTGCC AACCAAGCTTC CCTGGCCCCAT
 4681 TCTATGTTCA CCAGTGGTTG GGGTGCTTCT CCTGGCCCTT GGGGCCCTTC TCGTCCCTCCA
 4741 GCTCATTCGG CGACGGCGAC GAGAACATGG GGCCCTGTGG CTGCCCCCTG GTTTCATTCG
 4801 AAGGCTTCAG ACACAGCAGG CACCCCAACC GCGAGGCCCC CCACTGGGCG AGGACAACAT
 4861 TGGTCTTAAG GCACTGAAGC CAGAGGCCGA AGTGGATGAG GATGGAGTGG CCATGTGCTC
 4921 GGGCCCTGAA GAGGGAGAGG CTGAAGAAAC AGCCTCAGCC TCCAGGTGCC AGCTTTGGCC
 4981 GCTCAACAGC GGCTGTGGAG AGCTCCCCCA GGCAGCCATG CTGACCCCTC CTCAGGAGTG
 5041 TGAATCGGAG GTTCTGGATG TGGACACCTG TGGACCTGAT GGGGTGACAC CCCTGATGTC
 5101 AGCCGCTCTC TGTGGGGGAG TGCAGTCCAC GACTGGGGCT AGTCCACAGA GACTGGGGCT

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FIG. 9D

5161 AGGAAATCTG GAACCTTGGG AACCACTGCT GGATAGAGGG GCCTGCCCCC AGGCTCACAC
5221 TGTGGGCACT GGAGAGACGC CTCTGCACCT AGCTGCCAGA TTCTCTCGGC CAACCGCTGC
5281 CCGCCGCCCTC CTTGAGGCTG GAGCCAACCC CAACCAAGCCA GACCGCGCTG GCGCACCCCC
5341 ACTTCACACT GCTGTGGCTG CCGACGCTCG GGAGGTTTGC CAGCTCCTAT TGGCCAGCAG
5401 ACAGACTACG GTGGACGCCC GCACAGAGGA CCGGACTACA CCTTTGATGC TGGCTGCCAG
5461 GCTGGCCGTG GAGGACCTGG TTGAAGAATT GATCGCAGCC CGAGCAGATG TAGGAGCCAG
5521 GGATAAAAGG GGA AAAACTG CACTGCACTG GGCCGCTGCT GTGAACAACG CCCGAGCCGC
5581 CCGCTCTCTC CTCCAGGCTG GAGCGGATAA AGATGCCCCAG GACAGTAGGG AACAGACGCC
5641 GCTTTTCCCTG GCAGCGCGG AAGGAGCCGT GGAGGTGGCG CAGCTGTTGC TGGAGCTCGG
5701 GCGGGCCCCG GACTGCGAG ACCAGGCCGG GCTGGCCCCA GGAGATGTGG CCCGCCAGCG
5761 CAGTCACTGG GACCTGCTAA CGCTGCTGGA AGGGGCTGGA CCGACTACGC AGGAGGCCCG
5821 TCGGCACGCA CGCACACGC CCGGGGGCGG GTCCGCCCCG CGCTGCCGGA CGCTGTCTGC
5881 GGGAGCGCGC CCGCGGGGG GCGGAGCCTG TCTGCAGGCT CGCACTTGGT CCGTGGACTT
5941 GGGAGCGCGC GGAGGGAAGG TGTATGCTCG CTGCCGGAGC CGATCTGGAA GCTGCGGAGG
6001 CCCACCCACG CGCGGCCGCA GGTCTCCGC GGGCTCCCGT GGACGACGCG GGGCTAGGGC
6061 ATCACAGGAT GACTGGCCTC GCGACTGGT GGCCCTGGAA GCCTGCGGT CCGCCTGCAG
6121 TCGCGCGATC CCGCCTCCCA CCCTGACCCG GTCCCCAGAA CGTGGATCCC CTCAGTTGC
6181 CTGGGGTCTT CCAGTTCACC AAGAGATTCC CTTAAACTCG GTTGTAAAGAA ATCTGAACATA
6241 GGCAGCTGCG TGGAAAGGAG GAAGCGACAC GTACGAGTCT GGAAGACTCC GGACTTTTAA
6301 GGCCAAAATA ACCGTTAAGC TCACCTGTCT CCCCATAGA GTATGCACAG CAATGGGAAG
6361 AGGGTTTAGG ATGTCCGGTT GAGATAGACC GTGATTTTCC TGGAAAATAG GGCAGCTTCA
6421 AGAGGACAAA GTTGATTTCG AGAATCCCTA AACTCTGGAA CCAAGAACTG TGGGCGAATT
6481 GGGGTAAAAA TGTTCTTGT GTATGGTTTC CCAAAGGAG CCTCTGCTAT CTACTGCCCA
6541 CAAGTAGCTG GCACTATTT ATTAAGCACC TACGATGTGC CGGGTGTTGT GTAGATGAAC
6601 AGTAAGTAAC CAGTGGCCCA TCCAGCTGAT GACTCCTTGC CCTCTCTCTG CCTCCCCACA
6661 AGGACACTGG TGCAGGG

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FIG. 10A

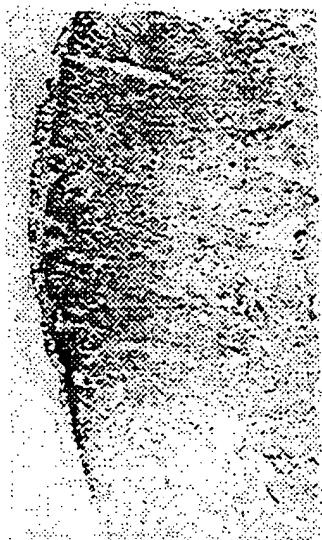


FIG. 10B

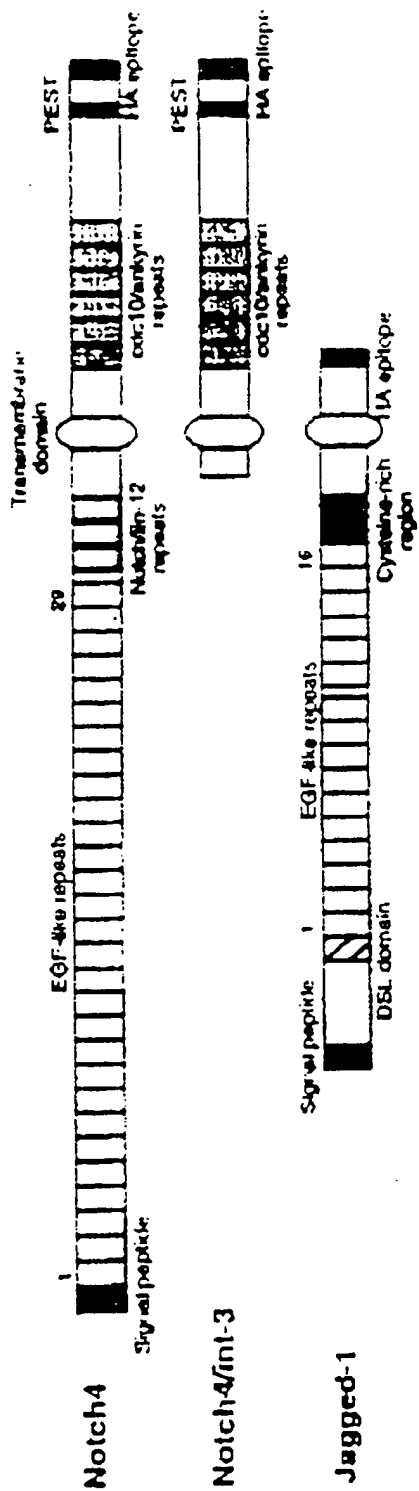


FIG. 10C



FIG. 10D

FIG. 11A



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FIG. 11D

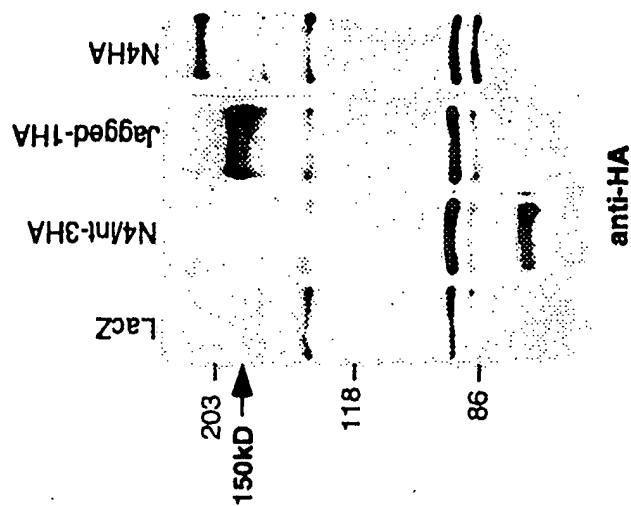


FIG. 11C

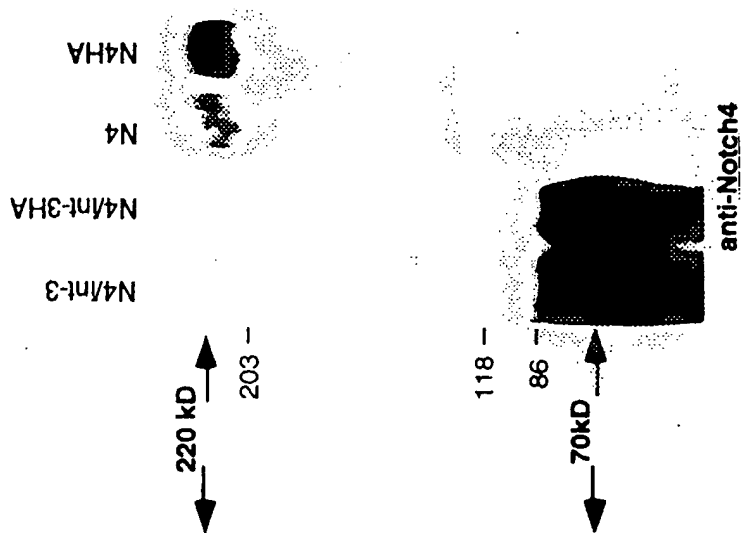
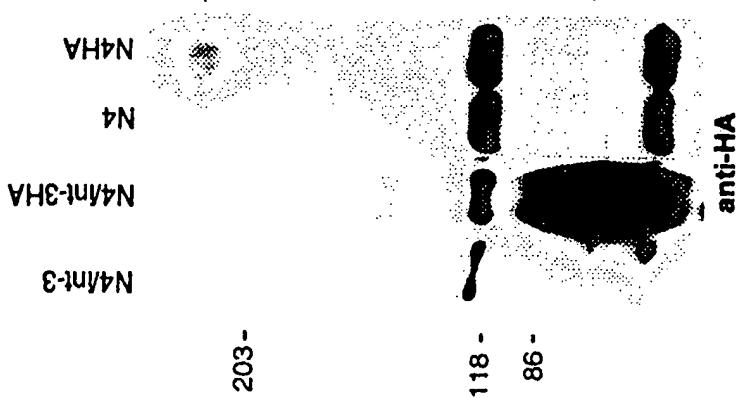


FIG. 11B



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FIG. 12A

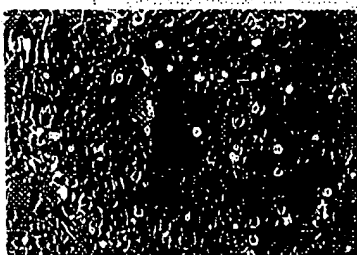


FIG. 12B

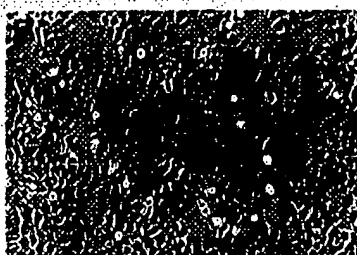


FIG. 12C

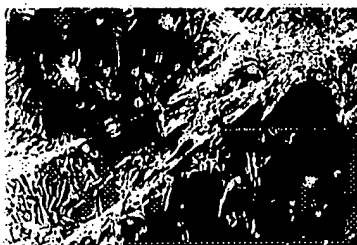


FIG. 12D

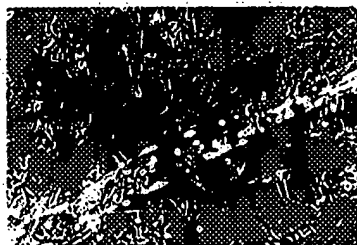


FIG. 12E

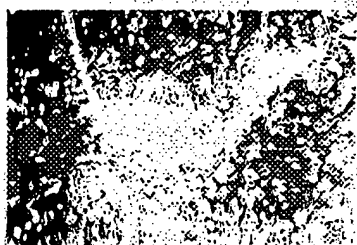


FIG. 12F



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FIG. 13A

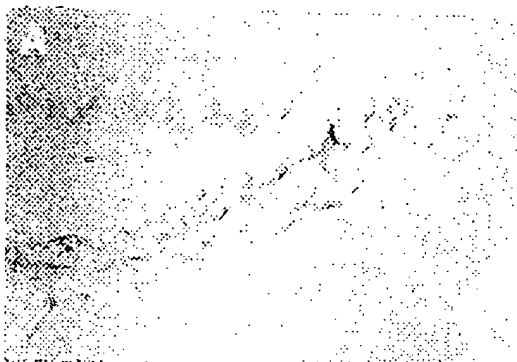


FIG. 13B

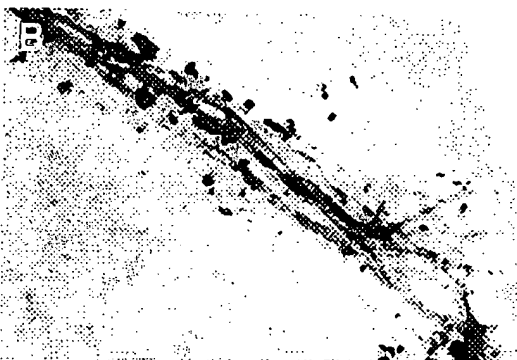
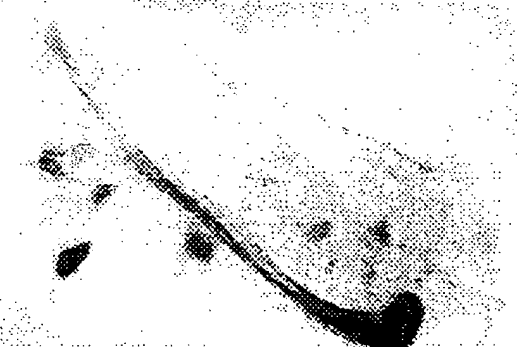


FIG. 13C



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FIG. 14A

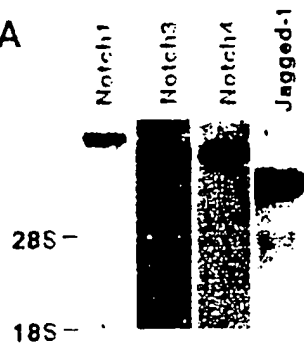
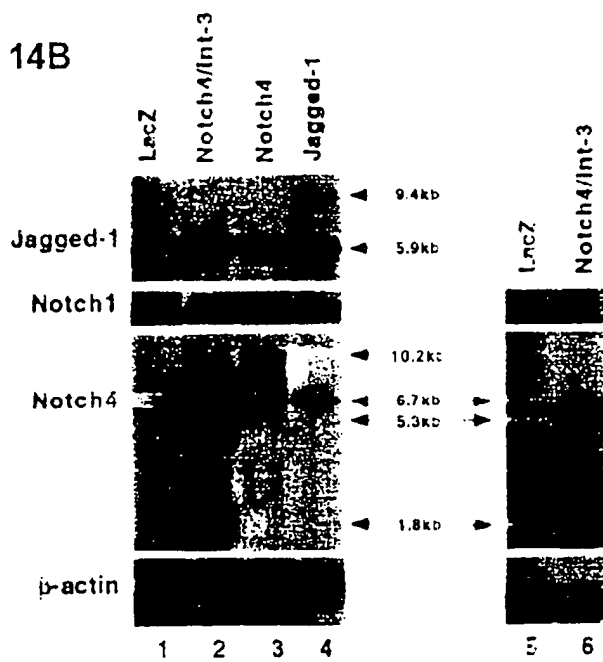


FIG. 14B



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/13050

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :Please See Extra Sheet.

US CL :Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/7.8, 29, 69.1, 172.3, 243, 320.1, 325, 375, 410; 514/1, 12, 44; 530/350, 387.1, 388.1; 536/23.5, 24.5

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|--------------|--|---|
| X -- Y | UYTTENDAELE et al., Notch4/int-3, a mammary proto-oncogene, is an endothelial cell-specific mammalian Notch gene. Development. July 1996, Vol. 122, No. 7, pages 2251-2259, see entire document, especially Figure 1. | 1-5, 9-23, 25 ----- 26, 28, 40-42 |
| X | SUGAYA et al., Three Genes in the Human MHC Class III Region near the Junction with the Class II: Gene for Receptor of Advanced Glycosylation End Products, PBX2 Homeobox Gene and a Notch Homolog, Human Counterpart of Mouse Mammary Tumor Gene int-3. Genomics. 15 September 1994. Vol. 23, No. 2, pages 408-419, see entire document, especially Figure 8. | 1-5, 9-23, 25 |

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

| | |
|---|--|
| * Special categories of cited documents: | *T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention |
| *A* document defining the general state of the art which is not considered to be of particular relevance | *X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone |
| *E* earlier document published on or after the international filing date | *Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art |
| *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) | *A* document member of the same patent family |
| *O* document referring to an oral disclosure, use, exhibition or other means | |
| *P* document published prior to the international filing date but later than the priority date claimed | |

Date of the actual completion of the international search

13 SEPTEMBER 1998

Date of mailing of the international search report

13 OCT 1998

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer

Terry A. McKelvey
TERRY A. MCKELVEY

Telephone No. (703) 308-0196

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/13050

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|-----------|---|-----------------------|
| X | SUGAYA et al., Gene organization of human NOTCH4 and (CTG) _n polymorphism in this human counterpart gene of mouse proto-oncogene Int3. Gene. 21 April 1997, Vol. 189, No. 2, pages 235-244, see entire document, especially Figure 3. | 1-5, 9-23, 25 |
| A | LINDELL et al., Jagged: A Mammalian Ligand That Activates Notch1. Cell. 24 March 1995, Vol. 80, pages 909-917, see entire document, especially Figure 1. | 1-42, 44-68 |
| Y | ZIMRIN et al., An Antisense Oligonucleotide to the Notch Ligand Jagged Enhances Fibroblast Growth Factor-induced Angiogenesis in Vitro. The Journal of Biological Chemistry. 20 December 1996, Vol. 271, No. 51, pages 32499-32502, see entire document, especially page 32500, column 2. | 26-28 |
| Y | JARRIAULT et al., Signalling downstream of activated mammalian Notch. Nature. 28 September 1995. Vol. 377. pages 355-358, see entire document, especially page 356, column 2. | 40-42 |
| X | ROBBINS et al., Mouse Mammary Tumor Gene int-3: a Member of the notch Gene Family Transforms Mammary Epithelial Cells. | 6-8, 24 |
| -- | | ----- |
| Y | Journal of Virology. April 1992, Vol. 66, No. 4, pages 2594-2599, see entire document, especially Figure 2. | 27 |

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/13050

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☒ Claims Nos.: 43
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

Please See Extra Sheet.

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐
☐

- The additional search fees were accompanied by the applicant's protest.
No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US98/13050

A. CLASSIFICATION OF SUBJECT MATTER:

IPC (6):

A61K 31/00, 31/70, 38/17; C07K 14/47, 16/00; C12N 1/00, 5/00, 5/10, 15/12, 15/63, 15/64; C12P 21/02; C12Q 1/02; G01N 33/53

A. CLASSIFICATION OF SUBJECT MATTER:

US CL :

435/7.8, 29, 69.1, 172.3, 243, 320.1, 325, 375, 410; 514/1, 12, 44; 530/350, 387.1, 388.1; 536/23.5, 24.5

B. FIELDS SEARCHED

Electronic data bases consulted (Name of data base and where practicable terms used):

APS, DIALOG (Biotech OneSearch databases)

search terms: notch4, pbs-notch4, antibod?, antisense, ligand, agonist, antagonist, assay?, measur?, screen?, jagged, angiogenesis, inhibit?, promot?, activated, int-3, monoclonal, pharmaceutical?, drug?.

BOX 1. OBSERVATIONS WHERE CLAIMS WERE FOUND UNSEARCHABLE

2. Where no meaningful search could be carried out, specifically:

Claim 43 claims a ligand that is a chemical not previously known to be a ligand which can specifically bind to Notch4 protein, identified by an assay comprising contacting Notch4 protein with the ligand under conditions permitting formation of specific complexes between Notch4 protein and known Notch4 protein-binding ligands and determining whether the ligand specifically binds to Notch4 protein. Because the identity or chemical structure of the ligands belonging to this genus are not known or suggested by the description, a search of the prior art would require a search for ligands of unknown structure, limited only by a specific function, specific binding to Notch4 protein. It is impossible to determine by searching the prior art if any of the literally millions of prior art ligands meet this functional limitation. Therefore, no meaningful search could be carried out for the claimed ligands.